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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/04, 38/10, 38/16, 38/19

A1

(11) International Publication Number: WO 99/12559

(43) International Publication Date: 18 March 1999 (18.03.99)

(21) International Application Number: PCT/US98/19216

(22) International Filing Date: 9 September 1998 (09.09.98)

(30) Priority Data:

60/058,352	9 September 1997 (09.09.97)	US
60/088,129	4 June 1998 (04.06.98)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	60/058,352 (CIP)
Filed on	9 September 1997 (09.09.97)
US	60/088,129 (CIP)
Filed on	4 June 1998 (04.06.98)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: INHIBITION OF APOPTOTIS USING PROSAPOSIN RECEPTOR AGONISTS

(57) Abstract

A method for inhibiting caspase-mediated apoptosis by administering prosaposin receptor agonists is provided. Apoptosis has a major causative role in diseases such as rheumatoid arthritis, irritable bowel syndrome, congestive heart failure, multiple sclerosis, Alzheimer's disease, Parkinson's disease, myocardial infraction, and coronary ischemia.

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INHIBITION OF APOPTOSIS USING PROSAPOSIN RECEPTOR AGONISTS

BACKGROUND OF THE INVENTION

1. *Field of the Invention*

This invention relates generally to apoptosis, and more specifically to the use of prosaposin receptor agonists to inhibit apoptosis upregulation of downstream cellular signaling molecules, such as Akt and Bcl-2 that act to inhibit caspase-mediated apoptosis.

2. *Background*

Prosaposin is the precursor of a group of four heat-stable glycoproteins that are required for glycosphingolipid hydrolysis by lysosomal hydrolases. Prosaposin, a 70 kilodalton (kDa) glycoprotein, is proteolytically processed to generate saposins A, B, C, and D. The saposins exist as 4 tandem domains in prosaposin before proteolysis. All 4 saposins are structurally similar to each other, having a similar placement of six cysteines, a glycosylation site and conserved proline residues. Unprocessed prosaposin also exists as an integral membrane protein and as a secreted protein that is present in human milk, cerebrospinal fluid and seminal plasma.

Prosaposin, saposin C, and prosaposin-derived peptides (prosaptides) have therapeutic applications in promoting functional recovery after toxic, traumatic, myocardial ischemic, degenerative and inherited lesions to the peripheral and central nervous system. *See,* U.S. Patent No. 5,571,787. Prosaposin and prosaptides can also be used to counteract the effects of demyelinating diseases by inducing neurite outgrowth stimulating myelination. The neurotrophic and myelinotrophic activity of prosaposin has been localized to amino acids 18-29 of saposin C.

Tumor necrosis factor α (TNFα) is a proinflammatory cytokine. TNFα induces a proinflammatory response in many disorders, including rheumatoid arthritis, Crohn's disease, irritable bowel syndrome, asthma, stroke cardiac infarction, and

congestive heart failure. After TNFα therapy was identified as a potential therapeutic target for rheumatoid arthritis, antibodies to TNFα were shown to be effective in both animal models and human patients. A similar approach was taken in animal models of inflammatory bowel disease. In another animal model of inflammatory pain, injection of TNFα into the subperineural space in the sciatic nerve immediately proximal to the sciatic notch produces neuropathic pain *in vivo*. The results of behavioral testing of either mechanical or thermal hyperalgesia showed that TNFα-injected animals displayed a significant hyperalgesia compared to vehicle-injected animals, in which hyperalgesia lasted for 5 days. Thus, the role of TNFα in various diseases has been established.

TNFα also induces programmed cell death (apoptosis) in several neural cell types, including cortical neurons, oligodendrocytes, and oligodendrocyte precursor cells. Apoptosis accounts for most of the programmed cell death in tissue remodeling and for the cell loss that accompanies atrophy of adult tissues following withdrawal of endocrine and other trophic factor stimuli. However, abnormal apoptosis is responsible for many human diseases after injury, including traumatic, chemical, myocardial ischemic, and genetic causes.

The proinflammatory cytokine interferon γ (IFNγ) is a potent inducer of oligodendrocyte apoptosis. Oligodendrocyte apoptosis has been observed at the advancing margins of chronic active multiple sclerosis (MS) plaques. IFNγ may therefore be a factor in the pathogenesis of multiple sclerosis by activating apoptosis in oligodendrocytes. Generally, proinflammatory cytokines such as TNFα and IFNγ are likely factors in the abnormal apoptosis underlying the pathogenesis in many demyelination disorders.

There is currently no effective treatment for the many diseases associated with abnormal apoptosis due to various causes.

SUMMARY OF THE INVENTION

The present invention provides a method for using prosaposin receptor agonists to inhibit apoptosis. Of particular interest is inhibition of apoptosis associated with caspase activation. Caspase activation resulting in apoptosis may be induced, for

example, by proinflammatory cytokines, as well as by Alzheimer's disease, stroke, myocardial ischemia, increased intracellular Ca^{++} levels, and increased levels of the neurotransmitter glutamate. The invention is thus useful for treating a proinflammatory cytokine-induced disease, such as multiple sclerosis, rheumatoid arthritis, irritable bowel syndrome, AIDS neuropathy and encephalitis, progressive multifocal leukoencephalitis, chronic myocardial atrophy, Alzheimers disease, and cell death of any type due to cytokine-induced apoptosis. One mechanism whereby prosaposin receptor agonists inhibit proinflammatory cytokine-induced apoptosis is by activation of the serine/threonine protein kinase Akt. Akt dissociates complexes of Bcl-2 family members, such as BAD-Bcl-2, releasing Bcl-2 and its family members which inhibit caspases, thereby inhibiting apoptosis. Thus, the activation (phosphorylation) of Akt by the action of prosaposin receptor agonists is a key event in the prevention of caspase-mediated apoptosis. The inhibition of apoptosis by prosaposin receptor agonists is a unique method of inhibiting apoptosis, because many other inhibitors of apoptosis inhibit caspase-mediated apoptosis at stages of the caspase proteolytic cascade different from the stage influenced by prosaposin receptor agonists. Thus, the use of prosaposin receptor agonists to inhibit caspase-mediated apoptosis represents a significant new function for these compositions.

An additional mechanism whereby prosaposin receptor agonists inhibit apoptosis is by blocking activation of JNK, a proapoptotic signaling component. Within several minutes after binding to the receptor, prosaposin receptor agonists block JNK activation induced by TNFα. The activation of JNK by TNFα is another well known mechanism for TNFα-induced, as well as other proinflammatory cytokine-induced, apoptosis.

The invention provides a method for inhibiting JNK-mediated and caspase-mediated apoptosis by contacting cells at risk of such apoptosis with an apoptosis-inhibiting amount of a prosaposin receptor agonist. The cells may be contacted *in vivo* or *ex vivo*. In one embodiment, the cells are oligodendrocytes, neurons, Schwann cells, or myocytes.

In another embodiment, the prosaposin receptor agonist has at least about 11 amino acids and comprises the amino acid sequence LeuIleXaa_1AsnAsnXaa_1Thr$Xaa_2$$Xaa_3$$Xaa_2$$Xaa_1$, wherein Xaa_1 is any amino acid; Xaa_2 is a charged amino acid; and Xaa_3 is optionally present and, when present, is a charged amino acid. In another embodiment, the prosaposin receptor agonist is a peptide selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is the polynucleotide sequence of human prosaposin cDNA.

FIG. 2 is the polypeptide sequences of prosaposin and saposin C.

FIG. 3 is the polypeptide sequence of several prosaposin-derived peptides.

FIG. 4 illustrates that the survival factor-promoted activation of Akt requires PI 3-kinase. Survival factor binding to the cognate receptor activates PI 3-kinase and other kinases. PI 3-kinase activates the serine/threonine kinase Akt. Subsequently, Akt phosphorylates specific targets, including the Bcl-2 family member BAD. Phosphorylation inactivates BAD, causing other BCL-2 family members to inhibit cell death (apoptosis) and allow cell survival. Survival factor binding to the cognate receptor also activates MAPK to promote cell survival.

FIG. 5 illustrates that prosaposin receptor agonist TX14(A) binding to prosaposin receptor acts to inhibit caspace-mediated apoptosis. Proinflammatory cytokine TNFα binds to TNF-R to activate adaptor molecules, such as TRADD. TRADD activates the caspase proteolytic cascade, causing apoptosis. Prosaposin receptor agonist binding to prosaposin receptor activates PI 3-kinase and other kinases. PI 3-kinase activates the serine/threonine kinase Akt. Subsequently, Akt phosphorylates specific targets, including the Bcl-2 family member BAD. Phosphorylation inactivates BAD, causing other BCL-2 family members to inhibit cell death (apoptosis) and allow cell survival.

FIG. 6 shows that (A) prosaposin and (B) prosaposin receptor agonist TX14(A) prevent TNFα-induced viability loss in NS20Y cells. NS20Y cells were incubated for 48 hr in DMEM containing 0.5% fetal bovine serum (FBS) and 100 ng/ml TNFα ± 2-fold dilutions of prosaposin (0 - 5 nM) or prosaptide (0 - 50 nM). Cell viability was assessed using MTT reduction. Results are mean ± SEM. Asterisk (*) indicates that mean is significantly different to TNFα treated cells; $p<0.05$.

FIG. 7 shows the time course of TNFα-induced viability loss and prevention by prosaposin receptor agonists. NS20Y cells were incubated in DMEM containing 0.5% FBS without (solid bar) or with (hatched bar) 100 ng/ml TNFα and 5 nM prosaposin (grey bar) or 50 nM prosaptide (hollow bar) for 48-96 hours. MTT was used to assess cell viability. Results are mean ± SEM. Asterisk (*) indicates that mean is statistically different to TNFα treated cells; $p<0.05$.

FIG. 8 shows that prosaposin receptor agonist TX14(A) prevents TNFα-induced death of NS20Y cells. NS20Y cells were treated with TNFα in DMEM containing 0.5% FBS with or without increasing doses of prosaptide. At 48 hr cells were stained with trypan blue to assess cell death. Results are mean ± SEM. C=control, T=TNFα.

FIG. 9 shows that prosaposin receptor agonist TX14(A) does not cause proliferation of NS20Y cells. NS20Y cells were seeded at 10,000/well in 96-well plates and grown in DMEM containing 0.5% FBS and 2-fold dilutions of prosaptide (P; solid bar) or in DMEM containing 2-fold dilutions of FCS (S; hatched bar). Cell proliferation was assessed at 48 hr by measuring BrdU incorporation. Data are mean ± SEM.

FIG. 10 shows inhibition of JNK2 phosphorylation in primary Schumann cells by a prosaposin derived peptide, TX14(A). Schwann cells were stimulated for 5 minutes with TNFα +/- TX14(A). Equal amounts of proteins from cell lysates were analyzed by SDS-PAGE and inununoblotted using a polyclonal antibody that recognizes phosphorylated JNK2 (Promega, Madison, WI). Proteins were detected by ECL (Amersham, Arlington Heights). Autoradiographs were scanned using ImageQuant™ (Molecular Dynamics, Sunnyvale, CA). Data are shown as representative data of two independent experiments.

FIG. 11 shows inhibition of p110 poly(ADP-ribose), PARP, cleavage by TX14(A). Primary Schwann cells were placed in low serum media (0.25% FBS) for 1 hour +/- TX14(A). Equal amounts of proteins from cell lysates were analyzed by SDS-PAGE and immunoblotted using a polyclonal antibody rhar recognizes PARP (Upstate Biotechnology, Lake Placid, NY). Proteins were detected by ECL (Amersham, Arlington Heights). Autoradiographs were scanned using ImageQuant™ (Molecular Dynamics, Sunnyvale, CA). Data are expressed as a mean ratio of p110 to p85 PARP ±SEM of two independent experiments.

FIG. 12 shows the effect of various doses of TX14(A) peptide (prosaptide; SEQ ID NO:7) on TNFα-induced Schwann cell death. The peptide concentration is shown on the x-axis and the percentage of trypan blue-stained cells is shown on the y-axis.

FIG. 13 shows the effect of prosaposin and TX14(A) (SEQ ID NO:7) on proinflammatory cytokine-induced cell death in undifferentiated CG4 oligodendrocytes. FIG. 13A shows the effect of prosaposin and TX14(A) (SEQ ID NO:7) on TNFα-induced cell death in undifferentiated CG4 oligodendrocytes. FIG. 13B shows the effect of prosaposin and TX14(A) on IFNγ-induced cell death in undifferentiated CG4 oligodendrocytes.

FIG. 14 shows that prosaposin receptor agonist TX14(A) inhibits proinflammatory cytokine TNFα-induced apoptosis in L6 myoblasts. L6 myoblasts cells were incubated for 96 hours either in media (control); media with 10 ng/ml TNFα (TNF Category); or media with 10 ng/ml TNFα and 200 ng/ml TX14(A). Cell death was measured by trypan blue assay.

FIG. 15 is a chart depicting the effect of prosaptide *in vivo* on thermal hyperalgesia following endoneurial injection of TNFα. Prosaptide (200 μg/kg) was injected subcutaneously 3 hr before injection of 10 μl TNFα (2.5 pg/ml).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for inhibiting apoptosis. At a fundamental level, the invention provides a method for inhibiting caspase-mediated apoptosis using a prosaptide, prosaposin, or saposin C. Caspases can be activated by several factors, including cytokines, anticancer drugs, growth factor deprivation, myocardial ischemia, metabolic toxins, and Ca^{++} toxicity. The method of the invention involves administering an apoptosis-inhibiting amount of a prosaposin receptor agonist to cells.

As used herein, the term "prosaposin receptor agonist" refers to a molecule that binds to any site on a cell to which prosaposin can bind, and to thereby alter the cell's function in the same manner to prosaposin. Examples of prosaposin receptor agonists include prosaposin, prosaptides, and saposin C. A receptor agonist is a substance that mimics the receptor ligand, is able to attach to that receptor, and thereby produces a same action that the ligand usually produces. Drugs are often designed as receptor agonists to treat diseases and disorders caused when the ligand, such as a hormone, is missing or depleted in a subject.

Prosaposin is a 70 kDa glycoprotein that is the precursor of a group of 4 small heat-stable glycoproteins that are required for hydrolysis of glycosphingolipids by lysosomal hydrolases. Prosaposin is a 517 amino acid protein, originally identified as the precursor of 4 sphingolipid activator proteins, as described in U.S. Patent No. 5,571,787. Four adjacent tandem domains in prosaposin are proteolytically processed in lysosomes to generate saposins A, B, C, and D, that activate hydrolysis of glycosphingolipids by lysosomal hydrolases. The unprocessed form of prosaposin is found in high concentrations in human and rat brain, where it is localized within neuronal surface membranes. During embryonic development, prosaposin mRNA is abundant in brain and dorsal root ganglia. Furthermore, prosaposin binds with high affinity to gangliosides, to stimulate neurite outgrowth, and promote transfer of gangliosides from micelles to membranes.

Prosaposin receptor agonists can be identified both structurally and functionally. A prosaposin receptor agonist has a structure that is similar to the region

of prosaposin that, when bound to the prosaposin receptor, induces a prosaposin receptor activity. For example, the prosaposin receptor agonist can have a structure that is similar to the amino acid sequence LeuIleXaa$_1$AsnAsnXaa$_1$ThrXaa$_2$Xaa$_3$Xaa$_2$Xaa$_1$, where Xaa$_1$ is any amino acid; Xaa$_2$ is a charged amino acid; and Xaa$_3$ is optionally present and, when present, is a charged amino acid. Functionally, a prosaposin receptor agonist induces a prosaposin receptor activity, for example, second messenger signaling, neurite outgrowth or myelination, decreased neuropathic pain, inhibition of proinflammatory cytokine-induced apoptosis, or inhibition of apoptosis caused by other agents.

In one embodiment, the prosaposin agonist is prosaposin itself. The prosaposin may be either prosaposin from native sources or prosaposin that is produced by recombinant methods, such as recombinant human prosaposin purified from spent media of *Spodoptera frugiperda* (Sf9) cells infected with a baculovirus expression vector containing full-length cDNA for human prosaposin. Human prosaposin has the amino acid sequence set forth in SEQ ID NO:2. The human cDNA sequence for prosaposin is SEQ ID NO:1. When the subject to be treated is human, human prosaposin and saposin sequence may more particularly be used.

In another embodiment, the prosaposin agonist is saposin C. The term "saposin C" refers to the proteolytic cleavage product from the third tandem domain of prosaposin. Saposin C can be isolated in pure form from spleens of patients with Gaucher disease, a lysosomal storage disorder, by the method of Morimoto *et al. (Proc. Natl. Acad. Sci. USA, 87:* 3493-3497, 1990). Human saposin C has the amino acid sequence set forth in SEQ ID NO:3.

The prosaposin agonist is a peptide including amino acids 18-28 of saposin C. The term "prosaptide" includes a peptide comprising amino acids 18-28 of saposin C (SEQ ID NO:4), peptides that have the activity of prosaptide comprising amino acids 18-28 of saposin C, or conservative variations of these amino acid sequences that retain a bioactivity of amino acids 18-28 of saposin C. A "conservative variation," as used herein, denotes the replacement of an amino acid residue by another, biologically similar residue. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the

substituted polypeptide also immunoreact with the unsubstituted polypeptide. Generally, only conservative amino acid alterations are undertaken, using amino acids that have the same or similar properties. Illustrative amino acid substitutions include the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine, glutamine, or glutamate; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; valine to isoleucine or leucine. Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant molecule without significantly altering its activity. This can lead to the development of a smaller active molecule. Such variations are encompassed by the present invention. An active octadecamer (18 amino acid) peptide fragment is set forth as SEQ ID NO:5. An active docosanamer (22 amino acid) peptide fragment is set forth as SEQ ID NO:6.

Thus, prosaptides of the invention have a length of at least about 11 amino acid residues, for example, at least about 14 amino acid residues. Prosaptides of the invention comprise about 80 or fewer amino acid residues, for example, no more than about 40 amino acid residues or no more that about 22 amino acid residues.

In another embodiment, the prosaposin receptor agonist is a prosaptide which has about 11 amino acids to about 80 amino acids (the full-length of saposin C) and the amino acid sequence LeuIleXaa$_1$AsnAsnXaa$_1$ThrXaa$_2$Xaa$_3$Xaa$_2$Xaa$_1$Xaa$_1$, where Xaa$_1$ is any amino acid; Xaa$_2$ is a charged amino acid; and Xaa$_3$ is optionally present and, when present, is a charged amino acid. For example, the prosaposin receptor agonist may be a prosaposin-derived peptide. The prosaposin receptor agonist may have the polypeptide sequence of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7. The polypeptide sequence LeuIleAspAsnAsnLysThrGluLysGluIleLeu (SEQ ID NO:4) corresponds to amino acids 18 to 29 of saposin C. The polypeptide sequence:

CysGluPheLeuValLysGluValThrLysLeuIleAspAsnAsnLysThrGluLysGluIleLeu

ID NO:6) corresponds to amino acids 8 to 29 of saposin C. The polypeptide sequence ThrDAlaLeuIleAspAsnAsnAlaThrGluGluIleLeuTyr (SEQ ID NO:7) corresponds to amino acids 16 to 29 of saposin C modified by a D-alanine for lysine substitution at position 2; an alanine for lysine substitution at position 8; a deletion of lysine at position 11 and the addition of a C-terminal tyrosine residue. *See,* TABLE 1. Such modifications can be useful for increasing peptide stability or uptake across the blood-brain barrier as described in EXAMPLE 6. As used herein, D-alanine can be represented by D-Ala or X.

TABLE 1

PEPTIDE	**SEQUENCE**	**SEQ ID NO:**
Prosaposin-derived 22-mer	CEFLVKEVTKLIDNNKTEKEIL	6
Prosaposin-derived 14-mer where X=D-alanine	TXLIDNNATE-EILY	7
Prosaposin-derived 11-mer	LIDNNKTEKEI	4

The prosaposin receptor agonist can also be an active fragment derived from another mammalian prosaposin. As used herein, the term "active fragment of prosaposin" is synonymous with "prosaptide." For example, an active fragment of mouse prosaposin, rat prosaposin, guinea pig prosaposin or bovine prosaposin such as SEQ ID NOS: 8 through 11 is a prosaposin receptor agonist.

The amino acid sequence of an active fragment of human prosaposin, that corresponds to amino acids 8 to 29 of saposin C (docosanomer; SEQ ID NO:6), is well conserved among other species, as shown in TABLE 2. In particular, adjacent asparagine (N) residues are conserved among human, mouse, rat, guinea pig and bovine prosaposins. In addition, a leucine (L) residue is conserved 3 to 4 residues toward the N-terminus of the 2 asparagine residues and one or more charged residues (aspartic acid (D), lysine (K), glutamic acid (E) or arginine (R)) are conserved 2 to 8 residues toward the C-terminus of the 2 asparagine residues. Each of these well-conserved residues is underlined in TABLE 2.

TABLE 2

SPECIES	SEQUENCE	SEQ ID NO:
Human	CEFLVKEVTKLIDNNKTEKEIL	6
Mouse	CQFVMNKFSELIVNNATE-ELLY	8
Rat	CQLVNRKLSELIINNATE-ELL	9
Guinea Pig	CEYVVKKVMLLIDNNRTEEKII	10
Bovine	CEFVVKEVAKLIDNNRTEEEIL	11

In another embodiment, the prosaposin receptor agonist is selected from a population of peptides related in amino acid sequence to SEQ ID NO:6 by having the conserved asparagine residues, a leucine/isoleucine residue, and one or more charged residues at the positions corresponding to the positions in which these residues are found in SEQ ID NO:6, but also having one or more amino acids that differ from the amino acids of SEQ ID NO:6.

A prosaposin receptor agonist can be identified by screening a large collection, or library, of random peptides or peptides of interest using assays that detect prosaposin receptor agonist function, for example, one of a number of animal models of apoptosis or inflammation known to those of skill in the art.

A prosaposin receptor agonist can be isolated or synthesized using methods well known in the art. Such methods include recombinant DNA methods and chemical synthesis methods for production of a peptide. Recombinant methods of producing a peptide through expression of a nucleic acid sequence encoding the peptide in a suitable host cell are well known in the art and are described, for example, in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Ed., Vols. 1 to 3, Cold Spring Harbor Laboratory Press, New York, 1989).

A prosaposin receptor agonist also can be produced by chemical synthesis, for example, by the solid phase peptide synthesis method of Merrifield *et al.* (*J. Am. Chem. Soc. 85*:2149, 1964). Standard solution methods well known in the art also can be used to synthesize a peptide useful in the invention. *See, for example,* Bodanszky (*Principles of Peptide Synthesis*, Springer-Verlag, Berlin, 1984) and Bodanszky (*Peptide

Chemistry, Springer-Verlag, Berlin, 1993). The chemically synthesized peptide may be prepared on an Applied Biosystems Model 430 peptide synthesizer using an automated solid-phase protocol provided by the manufacturer. Peptides may then be purified by high performance liquid chromatography (HPLC) on a Vydac C4 column to an extent greater than 95%. A newly synthesized peptide can be purified, for example, by high performance liquid chromatography (HPLC), and characterized using, for example, mass spectrometry or amino acid sequence analysis.

A particularly useful modification of a prosaposin receptor agonist is one that confers, for example, increased stability, by incorporation of one or more D-amino acids or substitution or deletion of lysine can increase the stability of a prosaposin receptor agonist by protecting against peptide degradation. For example, as disclosed herein, the prosaposin-derived tetradecamer SEQ ID NO:7 has an amino acid sequence derived from amino acids 16 to 29 of saposin C, but which has been modified by substitution or deletion of each of the 3 naturally occurring lysines and the addition of a C-terminal tyrosine residue. In particular, the prosaposin-derived tetradecamer SEQ ID NO:7 has a D-alanine for lysine substitution at position 2; an alanine for lysine substitution at position 8 and a deletion of lysine at position 11. The D-alanine substitution at position 2 confers increased stability by protecting the peptide from endoprotease degradation, as is well known in the art. *See, for example,* Partridge *(Peptide Drug Delivery to the Brain,* Raven Press, New York, 1991, page 247). The substitution or deletion of a lysine residue confers increased resistance to trypsin-like proteases, as is well known in the art. *See,* Partridge, *supra.* These substitutions increase stability and, thus, bioavailability of peptide SEQ ID NO:7, but do not affect activity in inhibiting apoptosis. The prosaposin receptor agonist can also be made as a cyclic peptide for increased stability.

A useful modification to a prosaposin receptor agonist can also be one that promotes peptide passage across the blood-brain barrier, such as a modification that increases lipophilicity or decreases hydrogen bonding. For example, a tyrosine residue added to the C-terminus of the prosaposin-derived peptide (SEQ ID NO:7) increases hydrophobicity and permeability to the blood-brain barrier. *See, for example,* Banks *et al.* *(Peptides 13:*1289-1294, 1992) and Partridge, *supra.* A chimeric

peptide-pharmaceutical that has increased biological stability or increased permeability to the blood-brain barrier, as described in EXAMPLE 6, for example, also can be useful in the method of the invention.

The term "prosaposin receptor" refers to a site on a cell to which prosaposin or a prosaposin receptor agonist can bind, thereby acting to alter the cell's function. The prosaposin receptor is a G-protein-coupled cell surface receptor of 54-60 kDa, isolated from baboon brains, pig brains, whole rat brain, and mouse neuroblastoma cells. This receptor protein can be isolated from a P100 plasma membrane fraction by affinity purification using a neurite growth-inducing peptide contained within the saposin C sequence linked to a solid support. The 54-60 kDa protein crosslinks irreversibly to saposin C. The isolation of the putative prosaposin receptor is described in EXAMPLES 6 and 7.

The term "apoptosis" refers to the cellular process of programmed cell death. Apoptosis encompasses a group of characteristic structural and molecular events, in which a cell specifically and precisely controls its fate in a mixed cell population. Endogenous nucleases cleave chromatin between nucleosomes and reduce the content of intact DNA in cells undergoing apoptosis. Apoptosis accounts for most of the programmed cell death in tissue remodeling and for the normal cell loss that accompanies atrophy of adult tissues following withdrawal of endocrine and other growth stimuli. Thus, apoptosis is similar to proliferation in that both processes are tightly regulated and essential for the homeostasis of renewable tissues. Apoptosis is also responsible, however, for the abnormal cell death that occurs in many diseases.

Apoptosis can be recognized by a characteristic pattern of morphological, biochemical and molecular changes in apoptotic cells. These changes can be broadly assigned to 3 stages: In the early stage, there is decreased cell size (cell dehydration), alterations in cell membranes, large (50 kilobase [kb]) DNA strand breaks, and an increase in cellular calcium levels. In the intermediate stage, DNA is cleaved into 180-200 bp fragments, giving the characteristic "laddering" on a DNA gel, further decrease in cell size, and a decreased cell pH. In the late stage, there is a loss of membrane function and the formation of apoptotic bodies.

Methods of detecting apoptosis can be based on the measurement of DNA content, altered membrane permeability, or the detection of endonucleolysis as characterized by DNA strand breaks. Such techniques are well known to those of skill in the art and can be readily performed without undue experimentation.

The apoptotic process can also be assayed by determining the activity of prosaposin receptor, Akt, Bcl-2 family members, associated PI 3-kinase pathway components, and JNK. Among the research tools that can be used are the well-known techniques involving antibodies and the various technologies (i.e., immunoprecipitation, immunoblotting, and immunoaffinity chromatography) that use these molecular probes. *See*, Kohler *et al.* (*Nature 256:* 495, 1975); *Current Protocols in Molecular Biology* (Ausubel *et al.*, ed., 1989); and Harlow and Lane (*Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1997). When used appropriately, these tools provide the means to analyze the activity of enzymes, identify post-translationally modified proteins, quantitate protein and non-protein macromolecules, and dissect the biochemical events of the many phases of the cell cycle. Phosphorylation assays, kinase activity assays, immunoprecipitations, and immunoassays are provided in EXAMPLE 1 and EXAMPLE 3.

The term "caspase" refers to any of the aspartate-specific cysteine proteases, sharing a conserved active site that cleaves proteins at a highly specific site, to induce apoptosis. All caspases cleave their substrates after aspartate (Asp) residues. Caspases promote apoptosis through proteolytic degradation of cellular components, a process which is amplified by autocatalysis of the various caspases. Different members of the caspase superfamily (formerly known as the ICE family) have slightly different substrate specificities and may thus be involved in different aspects of the apoptotic pathway. Caspases generally function in the distal portions of the proteolytic cascades involved in apoptosis (*see*, FIG. 5).

Caspases are processed from a single-chain zymogen to a two-chain active enzyme by cleavage at internal Asp residues. Caspases with large prodomains are generally regulatory caspases, whereas those with small prodomains are generally effector caspases. Thus, active caspases can activate other caspases following an initial

activating stimulus to form a proteolytic cascade, with the initial activation of a regulatory caspase serving to activate by proteolytic cleavage the downstream effector caspases.

During apoptosis, caspases break down cellular proteins, causing severe morphological changes and cell shrinkage. Effector caspases, particularly caspase-3, cleave substrates such as poly(ADP-ribose) polymerase, actin, fodrin, and lamin. In the final stages of apoptosis, the chromosomal DNA is cleaved by a DNase enzyme. The enzyme caspase-3 activated DNase (CAD) cleaves chromosomal DNA. CAD does not control apoptosis itself. Rather, the CAD inhibitor of caspase-3 activated DNase (ICAD) acts as a chaperone for CAD during CAD synthesis, remaining complexed with CAD to inhibit CAD DNase activity until the reactivity is triggered by appropriate apoptotic stimuli. Caspase-3, when activated by apoptotic stimuli, cleaves ICAD to release the DNase activity, allowing CAD, which carries a nuclear-localization signal, to enter the nucleus, and degrade chromosomal DNA in nuclei, causing the characteristic DNA fragmentation. *See*, Sakahira *et al.* (*Nature 391(6662):* 96-99, 1998); and Enari *et al.* (*Nature 391(6662):* 43-50, 1998). Thus, activation of CAD downstream of the caspase cascade is responsible for characteristic DNA degradation during apoptosis.

The method of the invention involves administering an apoptosis-inhibiting amount of a prosaposin receptor to cells. In one embodiment, the invention provides a method for inhibiting caspace-mediated apoptosis due to a proinflammatory cytokine. More particularly, the proinflammatory cytokine that induces apoptosis may be TNFα, one of the most intensively studied caspase activators. TNFα induces apoptosis in many cell types, including neurons, oligodendrocytes and oligodendrocyte precursor cells. While TNFα has been known to be important in proinflammatory responses for 20 years, the particular biochemical steps have been incompletely understood until recently. The TNFα is now a paradigm that has been applied to the other activators of caspace-mediated apoptosis.

TNFα effects are mediated through binding of TNFα to two types of receptor, the 75 kDa TNF-R1 and a 55 kDa TNF-R2. TNFα binding to a TNF-R initiates a variety of biological responses. For example, the cellular signaling in apoptosis begins at the

TNF-R1 and moves downstream in a series of biochemical reactions. Some biological responses, like cell proliferation and apoptosis, seem to be in opposition to each other, but TNF-R1 is known to control both kinds of biological responses. TNF-R1 has 3 separate responses: (1) apoptosis; (2) the activation of NF-κB, a transcription factor that inhibits apoptosis; and (3) the activation of JNK, a protein kinase.

The TNFα signal transduction pathway directly regulates caspase activation through recruitment of adaptor molecules and caspases to the cytoplasmic domain of TNF-R. TNF-R contains a cytoplasmic death domain (DD) that activates the apoptotic process by interacting with the DD-containing adaptor proteins TNF-R-associated DD protein (TRADD) and Fas-associated DD protein (FADD/MORT1), leading to the activation of cysteine proteases of the caspase family. The TRADD protein has two distinct functional domains. The protein has a DD body and a tail. The tail of TRADD binds to TRAF2, eventually resulting in activation of NF-κB. The body binds to FADD, another intracellular signaling protein, which then activates apoptosis. Another DD-containing protein that binds to TNF-R is caspase-8. Binding of TNFα stimulates TNF-R, leading to the formation of a receptor-bound death-inducing signaling complex (DISC), consisting of FADD and two different forms of caspase-8. As a result, activation of the caspase proteolytic cascade begins.

Another intensively studied caspase activator is Fas. Autoimmune disorders are associated with defects in Fas pathway function. Inappropriate expression of the Fas ligand (FasL) can enable tumor cells to escape immune surveillance. The Fas signal transduction pathway also directly regulates caspase activation through recruitment of adaptor molecules and caspases to the cytoplasmic domain of the receptor. Fas (Apo1; CD95) also contain a cytoplasmic death domain (DD) that activates the apoptotic process by interacting with TRADD and FADD, leading to caspase activation. Stimulation of Fas leads to the formation of a receptor-bound death-inducing signaling complex (DISC), consisting of FADD and two different forms of caspase-8. The discovery that Fas-associated death domain protein (FADD) recruited caspase-8 to the Fas signaling complex by virtue of caspase-8 ability to bind the adapter molecule FADD established that this protease has a role in initiating the death pathway.

The method of the invention, using a prosaposin receptor agonist is effective in inhibition of apoptosis in proinflammatory cytokine-susceptible cells that contain the prosaposin receptor, the downstream signaling elements of PI 3-kinase, Akt, and Bcl-2, and the caspase-mediated cell death mechanism (*See*, FIG. 4 and 5. *See also*, Hemmings, *Science 275*: 628-630, 1997; Franke *et al.*, *Nature 390*: 116-117, 1997; Datta *et al.*, *Cell 91*: 231-241, 1997).

Prosaposin receptor agonists stimulate several different signal transducers after binding to the prosaposin receptor. These signal transducers include mitogen-activated protein kinase (MAPK), PI 3-kinase, and the non-receptor tyrosine kinase $p60^{Src}$. Signal transduction following prosaposin receptor agonist binding to prosaposin receptor has been shown in neuronal cells, Schwann cells, and myoblasts. Prosaposin receptor agonists utilize a pertussis toxin sensitive G-protein pathway to activate MAPK proteins. Furthermore, Akt is upregulated within minutes of cellular exposure to prosaposin receptor agonist. Akt activates an apoptosis-inhibiting Bcl-2 family member, which then inhibits the action of caspases. Thus, prosaposin receptor agonists prevent caspase-mediated apoptosis.

Mitogen-activated protein kinase (MAPK) is a general name for a family of serine/threonine kinases that play an important role in cell signaling by a variety of ligands and receptors, including receptor tyrosine kinases and G-protein coupled receptors. The extracellular signal-regulated protein kinases, ERK1 and ERK2, are part of the MAPK family. ERK1 is the 44 kDa protein ($p44^{MAPK}$). ERK2 is the 42 kDa protein ($p42^{MAPK}$). Signaling proteins, such as phosphatidylinositol-3-kinase (PI 3-kinase) and protein kinase C (PKC) phosphorylate ERK proteins, either independently or in association with the guanosine triphosphate (GTP)-binding protein Ras ($p21^{Ras}$) pathway. In many cells, activation of the MAPK pathway by growth factors regulates gene transcription associated with proliferation and differentiation. In oligodendrocytes, ERK proteins also are important for oligodendrocyte process extension.

Prosaposin receptor agonists bind to the prosaposin receptor with high affinity to activate ERK1 and ERK2 phosphorylation in PC12 cells, Schwann cells, and

oligodendrocytes. Prosaposin receptor agonists also activate ERK activity by a pertussis toxin-sensitive mechanism involving the adapter protein Shc, $p60^{Src}$, and PI 3-kinase.

The survival of certain subsets of neurons of the peripheral nervous system can be promoted by the activation of a pathway that includes Ras and protein kinases leading to mitogen-activated protein kinase (MAPK). The PI 3-kinase pathway is also important for the survival of several cell lines. Activation of PI 3-kinase triggers the activation of the serine-threonine kinase Akt. Thus, Akt has a critical role in the PI 3-kinase pathway.

The activation of the serine/threonine protein kinase Akt is a key event in apoptosis prevention. Modules made of protein kinases control cellular processes, including apoptosis. After growth factors bind to their cognate growth factor receptor tyrosine kinases, PI 3-kinases are recruited and activated. Inositol lipids are phosphorylated by PI 3-kinases to act as second messengers. The serine/threonine protein kinase Akt (protein kinase B; PKB) is one of the major targets of PI 3-kinase-generated signals. Akt dissociates a complex of Bcl-2 family members, activating an apoptosis-inhibiting Bcl-2 family member, which then inhibits caspases to prevent apoptosis. Thus, the activation of Akt is a key event in cell death prevention by the PI 3-kinase pathway.

Akt is a proto-oncogene with a pleckstrin homology domain. The pleckstrin homology domains can bind lipids, providing a mechanism linking the activation of PI 3-kinase and Akt activity. PI 3-kinase activity can be inhibited by wortmannin and by the inhibitor LY294002. Both of these inhibitors inhibit the rapid activation of Akt by growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin, and insulin-like growth factor-1 (IGF-1). Activation of Akt by protein phosphatase inhibitors is, however, relatively insensitive to wortmannin and LY294002. Thus, the lipid kinase activity of PI 3-kinase mediates Akt activation by growth factors, so Akt acts downstream of PI 3-kinase (*see*, FIG. 4 and 5).

The PI 3-kinase-derived second messengers, phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4-P2) and, phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-PI 3), promote activation of Akt

in 3 steps: (i) the translocation of the kinase to the membrane, (ii) the attachment to the membrane by means of pleckstrin homology domain binding to phospholipid, and (iii) phosphorylation. The high-affinity association of Akt with PtdIns-3,4-P2 and PtdIns-3,2, promotes a conformational change leading to an increase of kinase activity. PtdIns-3,4-P2 and PtdIns-3,4-P3, which accumulate transiently upon cell stimulation by growth factors, also bind to the pleckstrin homology domain of Akt and promote the association of Akt with the membrane. PI 3-kinase activity further leads to an increase in Akt kinase by promoting Akt phosphorylation of Akt at 2 sites (Thr_{308} and Ser_{473}) by an upstream kinase, known as PKBK. Both phosphorylation events can be inhibited by wortmannin *in vivo*.

The release of Akt from the membrane by inositol trisphosphate (IP3) is the next regulatory step. IP3, generated from PtdIns-4,5-P2 by phospholipase C, releases pleckstrin homology domain-containing proteins, including Akt, from membranes. After its release, Akt becomes available to phosphorylate downstream targets.

Akt is particularly important for the survival of neurons. For example, IGF-1 protects cerebellar neurons from apoptosis by activating Akt. Nerve growth factor (NGF) also promotes Akt activation in pheochromocytoma PC12 cells, showing that kinase activation is also involved in the survival promoted by NGF. Thus, the Akt signaling pathway can prevent apoptosis of neurons.

The Akt signaling pathway for suppressing apoptosis then proceeds to the phosphorylation of the Bcl-2 family member BAD, thereby inactivating BAD promotion of apoptosis and promoting cell survival. *See,* Datta *et al.* (*Cell 91(2):* 231-24, 1997). Akt phosphorylates BAD *in vitro* and *in vivo* to inhibit BAD-induced apoptosis. The inactivation of BAD allows other, apoptosis-inhibiting Bcl-2 family members to inhibit caspases.

The mammalian Bcl-2 family has members that are potent inhibitors of programmed cell death and inhibit activation of caspases in cells (*e.g.*, Bcl-2, Bcl-x_L, and Bag). Other members of the Bcl-2 family promote apoptosis (*e.g.* Bax, Bcl-x_S, BAD, and Bak). However, Bcl-2 family members have several different mechanisms of function which need not be mutually exclusive. Bcl-2 family proteins either suppress or promote

apoptosis by interacting with and functionally antagonizing each other. The regulation of apoptosis by Bcl-2 family members involves several regulatory processes including dimerization and phosphorylation. Members of the Bcl-2 family form homodimers and heterodimers to interact with one another, altering the balance between cell survival and apoptosis. Phosphorylation can also change the activity state of many Bcl-2 family members. For example, phosphorylation of BAD by Akt causes BAD to be sequestered and inactivated.

All members of the Bcl-2 family share regions of homology termed BH (Bcl Homology) domains. The BH domains are (1) BH1 and BH2, which in apoptosis inhibitors allows heterodimerization with Bax to repress apoptosis; (2) BH3, which in the apoptosis promoters, Bax and Bak, allows heterodimerization with Bcl-x_L and Bcl-2 to promote apoptosis; and (3) BH4 which conserved in apoptosis inhibitors, *e.g.* Bcl-x_L, but absent in apoptosis agonists except Bcl-x_S. The BH4 domain allows interaction with apoptosis regulatory proteins such as Raf-1 and BAD. All members of the Bcl-2 family (except BAD and Bid) contain a hydrophobic C-terminus (transmembrane, TM) domain which anchors the Bcl-2 protein to the cell membrane. BAD lacks this sequence and is, therefore, located throughout the cytoplasm. Bcl-2 and Bcl-x_L localize predominantly to the outer mitochondrial membrane, but also to the nuclear and endoplasmic reticulum membranes. The GTP-binding protein Raf-1 translocates Bcl-2 family members to the mitochondrial membrane.

Dimerization of members of the Bcl-2 family regulates the cellular decision to proceed to apoptosis. Apoptosis-inhibiting members such as Bcl-2 and Bcl-x_L form dimers with the apoptosis-inducing activity of Bax and BAD to block Bax and BAD activity. Thus, the ratio of apoptosis-inhibiting Bcl-2 family members to apoptosis-inducing Bcl-2 family members is important in determining whether apoptosis will proceed. Excess apoptosis-inhibiting Bcl-2 family members promotes survival whereas excess apoptosis-inducing Bcl-2 family members promotes apoptosis. For example, Bcl-x_L homodimers are required to actively suppress apoptosis or to actively promote survival. Therefore, Bcl-x_L/Bax heterodimerization promotes apoptosis. By contrast, Bax homodimers are required to actively promote apoptosis or to actively inhibit survival.

Thus, Bcl-x_L/Bax heterodimerization inhibits apoptosis. If Bcl-2 levels are higher that those of Bax, for example, then survival generally prevails, whereas the opposite circumstance is associated with cell death. These interactions either prevent caspase activation to inhibit apoptosis, or promote caspase activation to induce apoptosis.

The Bcl-2 family member apoptosis inhibitors inhibit caspase activation. For example, there is a direct interaction between caspases and Bcl-x_L. The loop domain of Bcl-x_L is cleaved by caspases *in vitro* and in cells induced to undergo apoptotic death. Interaction of Bcl-x_L with caspases may be an important mechanism of inhibiting cell death. However, once Bcl-x_L is cleaved, the C-terminal fragment of Bcl-x_L potently induces apoptosis. Thus, the recognition/cleavage site of Bcl-x_L protects against apoptosis by acting at the level of caspase activation; cleavage of Bcl-x_L during the execution phase of programmed cell death converts Bcl-x_L from a protective to a lethal protein.

By inhibiting caspase activity in cells, prosaposin receptor agonists inhibit the apoptotic pathway and allow cell survival. Prosaposin receptor agonists inhibit apoptosis in proinflammory cytokine-susceptible cells that contain the prosaposin receptor, the downstream signaling elements of PI 3-kinase, Akt, and Bcl-2, and the caspase-mediated cell death mechanism. The inhibition of apoptosis by prosaposin receptor agonists occurs at the level of caspase activation, which is a unique method of inhibiting apoptosis. Known apoptosis inhibitors block caspace-mediated apoptosis at stages of the caspase proteolytic cascade different from the stage influenced by prosaposin agonists.

Most therapies do not inhibit apoptosis by inhibiting caspase through the activation of Akt and Bcl-2 family members. For example, many therapies involve the inhibition of the early stages of the apoptotic pathway by modulation of the binding of proinflammatory cytokines to their cognate receptors and the receptor activation. After TNFα therapy was identified as a potential therapeutic target for rheumatoid arthritis, antibodies to TNFα were shown to have efficacy in both animal models and human patients. *See,* Eigler *et al.* (*Immunol Today 18(10)*: 487-492, 1997). Studies in animals and an open-label trial have suggested a role for antibodies to TNFα, specifically the chimeric monoclonal antibody cA2, in the treatment of Crohn's disease. *See,* Targan *et*

al. (*N. Engl. J. Med.* *337(15)*: 1029-1035, 1997). Targan *et al.* found that single infusion of cA2 was an effective short-term treatment in many patients with moderate-to-severe, treatment-resistant Crohn's disease. Additionally, inhibiting proinflammatory cytokines (*e.g.*, TNFα and IL-1) is an established rheumatoid arthritis therapy. *See,* Maini *et al.* (*APMIS 105(4)*: 257-263, 1997). Clinical trials using monoclonal anti-TNFα antibodies have been particularly successful in controlling inflammation and markedly reducing acute phase proteins and cellular ingress. However, because disease invariably relapses, repeated therapy is necessary. Prosaposin receptor agonist treatment can be an effective alternative therapeutic agent for the treatment of Crohn's disease and rheumatoid arthritis because prosaposin receptor agonists are administered more easily than anti-TNFα antibodies, and animal studies demonstrate no antibodies against prosaptides after months of administration.

In certain therapies, activation of the transcription factor NF-κB inhibits apoptotic signaling through the transcriptional activation of survival-promoting genes. Prosaposin receptor agonists may also act through an alternative pathway to additionally promote survival.

In still other therapies, regulation of caspase-mediated apoptosis induction can be accomplished by expression of caspase inhibitors. Peptides that inhibit caspases are commercially available. For example, the peptides Caspase-3 Inhibitor I (DEVD-CHO; a highly specific, potent, reversible, and cell-permeable inhibitor of caspase-3); Caspase-3 Inhibitor III (Ac-DEVD-CMK; a potent, cell-permeable and irreversible inhibitor of caspase-3); and Caspase-4 Inhibitor I (Ac-LEVD-CHO; a caspase-4 inhibitor) are available from Calbiochem (San Diego, CA). Prosaposin receptor agonists do not compete with these peptides.

Prosaposin receptor agonists may also act to inhibit apoptosis through activation of a family of proteins known as Inhibitors of Apoptosis Proteins (IAPs). IAPs were first identified on the basis of sequence similarity to the insect baculovirus which infects cells and inhibits apoptosis. These molecules contain four conserved regions which have death antagonizing properties: (1) three baculovirus inhibitory repeats (BIR); and (2) a ring zinc finger domain. Both regions are likely involved in mediating

protein-protein interactions. One IAP gene, Neuronal Apoptosis Inhibitor Protein (NAIP), is selectively expressed in surviving neurons. NAIP was discovered to be the gene deleted in spinal muscular atrophy, a genetic disorder which causes spinal motor neuron degeneration and muscular atrophy leading to the death of newborn children. The IAPs act by preventing the activity or activation of caspases.

The process of identifying cells that are susceptible to caspase-mediated apoptosis may be accomplished in several ways, using laboratory methods known to those of skill in the art. For example, cells may be identified as susceptible to proinflammatory cytokine-induced apoptosis by one or a combination of the following analyses: identifying cells as undergoing apoptosis during events associated with contacting cells with proinflammatory cytokine; identifying proinflammatory cytokine during the events that cause cells to undergo apoptosis; preventing apoptosis by removing proinflammatory cytokine during the events that cause cells to undergo apoptosis; and inducing apoptosis by reintroducing proinflammatory cytokine or ending the removal of proinflammatory cytokine during the events that cause cells to undergo apoptosis. More particularly, cells may be identified as susceptible to proinflammatory cytokine-induced apoptosis by laboratory methods described by Vartanian *et al.* (*Molecular Medicine 1(7):* 732, 1995).

Expressly included as cells that are susceptible to proinflammatory cytokine-induced apoptosis are oligodendrocytes, neurons, Schwann cells, or myocytes. Since it is known that TNFα induces apoptosis in several neural cell types, including cortical neurons, oligodendrocytes and oligodendrocyte precursor cells, an identification of a cell as a neural cell is also an identification of that cell as susceptible to TNFα-mediated apoptosis. Since it is known that IFNγ induces oligodendrocyte apoptosis, an identification of a cell as an oligodendrocyte is also an identification of that cell as susceptible to IFNγ-mediated apoptosis.

Prosaposin receptor agonists are useful in treating diseases that involve cell death or that are mediated by proinflammatory cytokines. Prosaposin receptor agonists are therefore useful in treating degenerative diseases such as neurodegenerative diseases (*e.g.*, Alzheimer's disease, post-polio syndrome, Parkinson's disease, amyotrophic lateral

sclerosis, Huntington's disease), ischemic disease of the heart (*e.g.*, myocardial infarction), traumatic brain and spinal cord injury, pain syndromes, alopecia, AIDS, and toxin mediated liver disease. *See,* Nicholson *(Nature Biotechnology 14:* 297, 1996).

The identification of a subject having a proinflammatory cytokine-induced apoptotic disease can be accomplished by various methods known to those of skill in the art. For example, a disease may be identified as being a proinflammatory cytokine-induced disease by one or a combination of the following analyses: (1) identifying the disease as occurring during events associated with the proinflammatory cytokine; (2) identifying proinflammatory cytokine during the apoptotic disease events that cause inflammation; (3) preventing apoptosis by removing proinflammatory cytokine during the disease events; and (4) inducing apoptosis by reintroducing proinflammatory cytokine or ending the removal of proinflammatory cytokine during the disease events.

A subject to be treated according to the invention may be identified as being at risk for having a proinflammatory cytokine-induced disease at a future time. In EXAMPLE 6, the experimental subjects were identified as having a TNFα-mediated disease at the time of, or even before, the TNFα injection and therefore before the onset of hyperalgesia, a characteristic feature of experimental neuropathic pain states. The treatment method of the invention therefore includes both treating existing proinflammatory cytokine-induced disease and prophylactically reducing the severity of future proinflammatory cytokine-induced disease.

Prosaposin receptor agonists are therefore useful in treating many disorders in which TNFα is known to be involved, including rheumatoid arthritis (Feldman *et al.* *(Annals of the New York Academy of Sciences 766:* 272-278, 1995); Feldman *et al.* *(Journal of Inflammation 47:* 90-96, 1996)), Crohn's disease (Stokkers *et al.* *(Journal of Inflammation 47:* 97-103, 1996)), irritable bowel syndrome, asthma, stroke cardiac infarction, and congestive heart failure. *See.* Eigler *et al.* (*Immunol Today 18(10):* 487-492, 1997); MacLellan *et al.* (*Circ. Res. 81(2):* 137-144, 1997). TNFα induces apoptosis in several neuronal cell types, including cortical neurons, oligodendrocytes and oligodendrocyte precursor cells. The use of prosaposin receptor agonist for the treatment of any of these disorders is within the scope of the present invention. These agonists can

be administered either alone or as an adjunct to conventional anti-inflammatory therapies such as steroid administration.

The proinflammatory cytokine IFNγ is also a potent inducer of oligodendrocyte apoptosis. Oligodendrocyte apoptosis has been observed at the advancing margins of chronic active multiple sclerosis (MS) plaques (Vartanian *et al.*, *Molecular Medicine 1(7):* 732, 1995). IFNγ may therefore be a factor in the pathogenesis of multiple sclerosis by activating apoptosis in oligodendrocytes. The method of the invention may be used for halting or slowing the progress of the IFNγ-mediated diseases associated with neural or myelin degeneration in neural tissue, by contacting neuronal tissue susceptible to such degradation with a prosaposin receptor agonist. There are several diseases that result in inflammatory demyelination of nerve fibers including multiple sclerosis, Guillain-Barre disease, AIDS neuropathy, and AIDS cortical demyelination. These diseases can be treated by administration of prosaposin receptor agonists to the cells affected by the disease. Because the molecular weight of the active docosanamer (22 amino acid; SEQ ID NO:6) is approximately 2600, and an octadecamer (18 amino acid; SEQ ID NO:5) contained within this sequence will cross the blood-brain barrier, the docosanamer will also cross and enter the central nervous system. TNFα and IFNγ may further be factors in the death of oligodendrocyte cells which underly the pathogenesis in many demyelination disorders. A patient diagnosed as having a demyelination disease would also be expressly identified as having a proinflammatory cytokine-induced disease that may be treated by the method of the invention.

Prosaposin receptor agonists can also be used in the treatment of Alzheimer's disease. Caspase inhibition is relevent to neurodegenerative disease and inhibition of caspase activity may have an impact on the clinical course of neurodegenerative diseases, such as Alzheimer's disease. *See*, Holtzman *et al.* (*Nature Medicine 3(9):* 954-955, 1997). Prosaposin receptor agonists, especially prosaposin receptor agonists that cross the blood-brain barrier, treatment can be a therapeutically effective agent for treating neurodegenerative diseases of the central nervous system.

Administration of a prosaposin receptor agonist can provide an effective therapy for treatment of heart disease by inhibiting the effects of associated

proinflammatory cytokines. Apoptosis is a contributing cause of cardiac myocyte loss in ischemia/reperfusion injury, myocardial infarction, and long-standing heart failure. *See,* MacLellan *et al.* (*Circ. Res. 81(2):* 137-144, 1997). Insights into the molecular circuitry controlling apoptosis suggest the potential to protect heart muscle from apoptosis through one or more of these pathways by pharmacological means. Cytokines that are expressed within the myocardium in response to environmental injury, such as TNFα, IL-1, IL-6, are important for initiating and integrating homeostatic responses during cardiovascular disease. *See,* Mann (*Cytokine Growth Factor Rev. 7(4):* 341-354, 1996). For example, the failing human heart expresses TNFα. *See,* Kubota *et al.* (*Circ. Res. 81(4):* 627-635, 1997) in the development of congestive heart failure.

The ability of myocardium to successfully compensate for, and adapt to, stress ultimately determines whether the heart will decompensate and fail, or whether it will maintain preserved function. Thus, the myocardial response to environmental stress is very important to heart function. *See,* Mann (*Cytokine Growth Factor Rev. 7(4):* 341-354, 1996). Cytokines that are expressed within the myocardium in response to environmental injury, *i.e.,* TNFα, IL-1, and IL-6, are important for initiating and integrating homeostatic responses within the heart. However, these proinflammatory cytokines all can produce cardiac decompensation when expressed at sufficiently high concentrations. Accordingly, the short-term expression of proinflammatory cytokines within the heart may provide the heart with an adaptive response to stress, whereas long-term expression of proinflammatory cytokines are maladaptive by producing cardiac decompensation.

The arthritogenic activities of TNFα and its p55 TNF-R have been well documented in experimental animal models of arthritis and in transgenic mice expressing wild-type or mutant transmembrane human TNFα proteins in their joints. *See,* Alexopoulou *et al.* (*Eur. J. Immunol. 27(10):* 2588-2592, 1997). Prosaposin receptor agonist administration can provide an effective therapy for treatment of arthritis, because prosaposin receptor agonists inhibit the effects of proinflammatory cytokines downstream of the interactions between TNFα and TNF-R.

In summary, administering prosaposin receptor agonists to inhibit caspase-mediated apoptosis, includes the use of such agonists in the treatment of diseases such as rheumatoid arthritis, Crohn's disease, irritable bowel syndrome, asthma, cardiac infarction, congestive heart failure, multiple sclerosis, acute disseminated inflammatory (AIDS) leukoencephalitis, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, post-polio syndrome, Huntington's disease, ischemic heart disease, traumatic brain injury, traumatic spinal cord injury, alopecia, AIDS dementia, cerebral malaria, HTLV neuropathy, Guillain-Barre disease, AIDS neuropathy, inflammatory neurodegenerative diseases, and toxin-induced liver disease.

The term "apoptosis-inhibiting amount" means the amount of prosaposin receptor agonist needed to inhibit apoptosis in a target cell. The amount of prosaposin receptor agonist that inhibits apoptosis can easily be determined by one of skill in the art using standard methods for assaying apoptosis. The activity of a prosaposin receptor agonist in inhibiting apoptosis can correlate with neurotrophic activity and activity in alleviating neuropathic pain or inducing neurotrophic activity. For example, the prosaposin-derived docosanomer (SEQ ID NO:6) and the prosaposin-derived tetradecamer (SEQ ID NO:7) alleviate neuropathic pain and have neurotrophic activity. The prosaposin-derived dodecamer peptide (SEQ ID NO:4), which has the conserved adjacent asparagines, leucine and charged residues described above, is active as a neurotrophic factor. A typical minimum amount of prosaposin for the neurotrophic factor activity in cell growth medium is usually about 1.4×10^{-11} M, or about 10 ng/ml. This amount or more of prosaposin receptor agonists may also be used to inhibit apoptosis or reduce inflammation. Typically concentrations in the range of 0.1 μg/ml to about 10 μg/ml of any of these materials will be used.

The contact between the prosaposin receptor agonist and the cells may be performed *ex vivo* or *in vivo*. Cells can be treated *ex vivo* by directly administering prosaposin receptor agonists to the cells. For example, cells can be treated *ex vivo* by culturing the cells in growth medium suitable for the particular cell type followed by addition of the agonist to the medium. Such *ex vivo*-treated cells can then be administered to a patient.

Cells are treated *in vivo* by administering the agonist by any effective method that will result in contact between the prosaposin receptor agonist and the cell. The method of administration of an apoptosis-inhibiting amount of prosaposin receptor agonist may be by conventional modes of administration, including intravenous, intramuscular, intradermal, pulmonary, nasal, mucosal, subcutaneous, epidural, intraocular, topical in a biologically compatible carrier and oral administration. The composition may be injected directly into the blood in sufficient quantity to give the desired *in vivo* concentration. Direct intracranial injection or injection into the cerebrospinal fluid also may be used provided sufficient quantities can be given such that the desired local concentration is achieved. A pharmaceutically acceptable injectable carrier of well known type can be used. Such carriers include, for example, phosphate buffered saline (PBS) or lactated Ringer's solution. Alternatively, the composition can be administered to peripheral neural tissue by direct local injection or by systemic administration.

One skilled in the art can readily assay the ability of a prosaposin receptor agonist to cross the blood-brain barrier *in vivo*, for example, as disclosed in EXAMPLE 6. In addition, an active fragment of prosaposin can be tested for its ability to cross the blood-brain barrier using an *in vitro* model of the blood-brain barrier based on a brain microvessel endothelial cell culture system, for example such as that described by Bowman *et al.* (*Ann. Neurol. 14:*396-402, 1983) or Takahura *et al.* (*Adv. Pharmacol. 22:*137-165, 1992). It had long been believed that in order to reach neuronal populations in the brain, neurotrophic factors would have to be administered intracerebrally, since these proteins do not cross the blood-brain barrier. However, the active octadecamer (18 amino acid; SEQ ID NO:5) will cross and the active docosanamer (22 amino acid; SEQ ID NO:6) likely crosses this barrier and would thus contact the brain cells following intravenously administration. An octadecamer (18 amino acid; SEQ ID NO:5) peptide consisting of amino acids 12-29 of the docosanamer (SEQ ID NO:6) with a substitution of tyrosine for valine at amino acid 12 (with a molecular weight of 2 kDa) crosses the blood-brain barrier and enters the central nervous system. Conditions under which a

peptide can cross the blood-brain barrier and enter the nervous system are described by Banks *et al.* (*Peptides 13:* 1289-1294, 1992).

Other neuronal populations, such as motor neurons, also can be treated by intravenous injection, although direct injection into the cerebrospinal fluid is also envisioned as an alternate route.

Oral administration often is desirable, provided the prosaposin receptor agonist is resistant to gastrointestinal degradation and readily absorbable. The substitution, for example, of one or more D-amino acids can confer increased stability to a prosaposin receptor agonist useful in the invention. Retroinverso peptidomimetics that are stable and retain bioactivity can also be devised, as described by Brugidou *et al.* (*Biochem. Biophys. Res. Comm. 214(2):* 685-693, 1995) and Chorev *et al.* (*Trends Biotechnol. 13(10):* 438-445, 1995).

The prosaposin receptor agonists can be packaged and administered in unit dosage form such as an injectable composition or local preparation in a dosage amount equivalent to the daily dosage administered to a patient or as a controlled release composition. A septum sealed vial containing a daily dose of the active ingredient in either phosphate-buffered saline or in lyophilized form is an example of a unit dosage. Appropriate daily systemic dosages of agonist based on the body weight for treatment of caspase-mediated apoptosis are in the range of from about 10 to about 100 μg/kg, although dosages from about 0.1 to about 1,000 μg/kg are also contemplated. Thus, for the typical 70 kg human, a systemic dosage can be between about 7 and about 70,000 μg daily and, alternatively, between about 700 and about 7,000 μg daily. A daily dosage of locally administered material will be about an order of magnitude less than the systemic dosage.

A prosaposin receptor agonist also can be administered in an inhalant form. Inhalant drug delivery has been successfully used for β-agonist and corticosteroid drugs of emphysema. *See*, Pingleton (*JAMA*, June 19, 1996). Mask ventilation is now a first-line therapy for patients who have an exacerbation of chronic obstructive pulmonary disease. Similar methods of inhalant drug delivery can be used to deliver prosaposin receptor agonists.

A prosaposin receptor agonist also can be administered in a sustained release form. The sustained release of a prosaposin receptor agonist has the advantage of inhibiting apoptosis over an extended period of time without the need for repeated administrations of the active fragment. Sustained release can be achieved, for example, with a sustained release material such as a wafer, an immunobead, a micropump or other material that provides for controlled slow release of the prosaposin receptor agonist. Such controlled release materials are well known in the art and available from commercial sources (Alza Corp., Palo Alto CA; Depotech, La Jolla CA. *See also,* Pardoll *(Ann. Rev. Immunol. 13:* 399-415, 1995)). In addition, a bioerodible or biodegradable material that can be formulated with a prosaposin receptor agonist, such as polylactic acid, polygalactic acid, regenerated collagen, liposomes, or other conventional depot formulations, can be implanted to slowly release the active fragment of prosaposin. The use of infusion pumps, matrix entrapment systems, and transdermal delivery devices also are contemplated in the present invention.

The invention also provides a method for inhibiting apoptosis or alleviating inflammation in a subject by transplanting into the subject a cell genetically modified to express and secrete a prosaposin receptor agonist. Transplantation can provide a continuous source of a prosaposin receptor agonist and, thus, sustained alleviation of neuropathic pain. For a subject suffering from prolonged apoptosis, such a method has the advantage of obviating or reducing the need for repeated administration of an active fragment of prosaposin.

Using methods well known in the art, a cell can be readily recombinantly modified, such as by transfection with an expression vector containing a nucleic acid encoding a prosaposin receptor agonist. *See,* Chang (*Somatic Gene Therapy,* CRC Press, Boca Raton, 1995). Following transplantation into the brain, for example, the transfected cell expresses and secretes a prosaposin receptor agonist and, thus, inhibits apoptosis. Such a method can be useful to alleviate neuropathic pain as described for the transplantation of cells that secrete substances with analgesic properties. *See, for example,* Czech and Sagen *(Prog. Neurobiol. 46:*507-529, 1995). In practice, the transfected cell should be immunologically compatible with the subject. Consequently,

autologous cells are particularly useful for recombinant modification. Non-autologous cells also can be useful if protected from immune rejection using, for example, microencapsulation or immunosuppression. Useful microencapsulation membrane materials include alginate-poly-L-lysine alginate and agarose (*See, for example,* Goosen (*Fundamentals of Animal Cell Encapsulation and Immobilization,* CRC Press, Boca Raton, 1993); Tai and Sun (*FASEB J. 7:*1061, 1993); Liu *et al. (Hum. Gene Ther. 4:*291, 1993); and Taniguchi *et al. (Transplant. Proc. 24:* 2977, 1992)).

For treatment of a human subject, the cell can be a human cell, although a non-human mammalian cell also can be useful. In particular, a human fibroblast, muscle cell, glial cell, neuronal precursor cell or neuron can be transfected with an expression vector to express and secrete an active fragment of prosaposin such as SEQ ID NO:4. A primary fibroblast can be obtained, for example, from a skin biopsy of the subject to be treated and maintained under standard tissue culture conditions. A primary muscle cell also can be useful for transplantation. Considerations for neural transplantation are described, for example, in Chang, *supra.*

A cell derived from the central nervous system can be particularly useful for transplantation to the central nervous system, since the survival of such a cell is enhanced within its natural environment. A neuronal precursor cell is particularly useful in the method of the invention since a neuronal precursor cell can be grown in culture, transfected with an expression vector and introduced into an individual, where it is integrated. The isolation of neuronal precursor cells that are capable of proliferating and differentiating into neurons and glial cells is described in Renfranz *et al. (Cell 66:*713-729, 1991).

Methods of transfecting cells *ex vivo* are well known in the art. *See,* Kriegler (*Gene Transfer and Expression: A Laboratory Manual,* W.H. Freeman & Co., New York, 1990). For the transfection of a cell that continues to divide, such as a fibroblast, muscle cell, glial cell or neuronal precursor cell, a retroviral vector is preferred. For the transfection of an expression vector into a postmitotic cell such as a neuron, a replication-defective herpes simplex virus type 1 (HSV-1) vector is useful. *See,* During

et al. (*Soc. Neurosci. Abstr.* *17*:140, 1991) and Sable *et al.* (*Soc. Neurosci. Abstr.* *17*:570, 1991).

A nucleic acid encoding an active fragment of prosaposin can be expressed under the control of one of a variety of promoters well known in the art, including a constitutive promoter or inducible promoter. *See, for example,* Chang, *supra.* A particularly useful constitutive promoter for high level expression is the Moloney murine leukemia virus long-terminal repeat (MLV-LTR), the cytomegalovirus immediate-early (CMV-IE) or the simian virus 40 early region (SV40).

The invention provides a method of alleviating neuropathic pain by administering a neuropathic pain-alleviating amount of a prosaposin receptor agonist to a subject who is suffering from neuropathic pain caused by proinflammatory cytokine. The invention is therefore useful for treating neuropathic pain component of inflammatory disease, although the pain relief is not due to an anti-inflammatory effect. The prosaposin receptor agonist activation of Akt described *supra* for inhibition of caspace-mediated apoptosis is relevant to the alleviation of neuropathic pain.

In an animal model, injection of TNFα into the subperineural space in the sciatic nerve immediately proximal to the sciatic notch produces neuropathic pain *in vivo*. *See,* Wagner *et al.* (*NeuroReport* *7*: 2897-2901, 1996). Using behavioral testing of either mechanical or thermal hyperalgesia, TNFα-injected animals suffer significant hyperalgesia compared to vehicle-injected animals, whose algesia lasted for 5 days. The pain is due to nerve damage. Administration of prosaposin receptor agonist prevents the thermal hyperalgesia that occurs upon injection of TNFα into the sciatic nerve. An example of the alleviation of neuropathic pain by prosaposin receptor agonist in the rat TNFα-injection model is provided in EXAMPLE 6.

A reduction in pain can be determined by behavioral measurements assessing the response to thermal or mechanical stimulii. When the subject is human, the subject can report a reduction in pain. Reduction in pain can also be detrmined in animal models. Several animal models of neuropathic pain have been developed, including the Chung rat model, the streptozotocin-induced insulin-deficient diabetic rat model, the Seltzer rat model, the neuroma model, and several primate models. *See,* Myers (*NIH Workshop on*

Low Back Pain (J. Weinstein, S. Gordon (Eds), American Academy of Orthopaedic Surgeons, 1995)); Myers (*Regional Anesthesia 20(3):* 173-184, 1995) and Bennett (*Muscle & Nerve 16:*1040-1048, 1993). The scientific literature on nerve root injury has expanded recently with the introduction of new models of *cauda equina* compression. Because common pathogenic mechanisms of nerve and nerve root injury are associated with the development of chronic pain states, an alleviation of neuropathic pain by administration of prosaposin receptor agonist that is successful in any one animal model of neuropathic pain may be extrapolated to all models and to all types of human neuropathic pain, as prosaposin receptor agonists operate at a fundamental convergent step in the pathogenesis of pain arising from nerve injury. An effective concentration of prosaposin receptor agonist may also be determined by comparison with the concentrations of prosaposin receptor agonist recommended for other conditions.

Activation of immune cells by pathogens also induces the release of a proinflammatory cytokines. *See,* Watkins *et al.* (*Brain Res. 692(1-2):* 244-250, 1995). The activated immune system communicates to the brain by release of proinflammatory cytokines. *See,* Watkins *et al.* (*Pain 63(3):* 289-302, 1995). Proinflammatory cytokines mediate a variety of common neuropathic pain states. Illness responses in the brains of those suffering from neuropathic pain cause dramatic changes in neural functioning. For example, IL-1β can alter brain function, resulting in a variety of illness responses including increased sleep, decreased food intake, fever, *etc.* IL-1β also produces neuropathic pain. This IL-1β-induced neuropathic pain is mediated by activation of subdiaphragmatic vagal afferents in the brain.

The physiological basis of IL-1β-induced neuropathic pain is representative of a general physiological basis for proinflammatory cytokine-induced neuropathic pain. For example, TNFα produces dose-dependent neuropathic pain as measured by the tailflick test. This TNFα-induced neuropathic pain is further mediated by the induced release of IL-1β. Furthermore, this TNFα-induced hyperalgesia (as well as most illness responses) is also mediated by activation of subdiaphragmatic vagal afferents. The effects of subdiaphragmatic vagotomy cannot be explained by a generalized depression of neural excitability. *See,* Watkins *et al.* (*Brain Res. 692(1-2):* 244-250, 1995).

Proinflammatory cytokines and the neural circuits that they activate are therefore involved in the neuropathic pain states produced by irritants, inflammatory agents, and nerve damage.

Thus, apparently diverse neuropathic pain states converge in the central nervous system and activate similar or identical neural circuitry. Prosaposin receptor agonists that cross the blood-brain barrier are especially useful for treatment of illness response and other proinflammatory cytokine-induced neuropathic pain components in the central nervous system.

The following examples are illustrative and are not intended to limit the scope of the present invention.

EXAMPLE 1

PROSAPOSIN RECEPTOR AGONISTS PREVENT TNFA-INDUCED DEATH OF A NEURONAL CELL LINE

The purpose of this EXAMPLE was show that prosaposin and a peptide derived from prosaposin could prevent TNFα neurotoxicity. TNFα treatment for 48 hr or more caused up to 50% loss of viability in a neuronal cell line, NS20Y, as demonstrated by MTT reduction. Prosaposin and prosaptide TX14(A) prevented the loss of viability dose dependently, with maximal protection seen at 5 nM and 50 nM, respectively. Trypan blue exclusion and BrdU incorporation assays showed that prosaptide increased viability by preventing cell death and did not cause cell proliferation. The prevention of TNFα-induced death by prosaposin receptor agonists was not inhibited by pertussis toxin. Thus, the results of this EXAMPLE show that prosaposin and the prosaptide TX14(A) prevented the death of a neuronal cell line induced by TNFα by a pertussis toxin-insensitive pathway.

Materials and Methods. Prosaposin was purified from human milk as previously described by Hiraiwa *et al.* (*Arch. Biochem. Biophys. 304:* 110-116, 1993).

Prospatide (TX14(A), was provided by Anaspec (San Jose, CA) at greater than 95% purity. TNFα was purchased from R&D Systems (Minneapolis, MN) and pertussis toxin (PT) was from Calbiochem (San Diego, CA). Cell culture reagents were purchased from Gibco-BRL (Grand Island, NY).

The mouse neuroblastoma cell line, NS20Y, was a gift from Drs. T. Taketomi and K. Uemura (Shinshu University, Matsumoto, Japan). Cells were maintained in DMEM (high glucose) containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 1.1 mg/ml sodium pyruvate, at 37°C under humidified 5% CO_2.

For cell viability assays, cells were seeded at $1x10^4$/well in 96-well plates in complete media and allowed to grow overnight. The next day, TNFα and prosaposin or prosaptide were applied to cells in DMEM containing penicillin, streptomycin, sodium pyruvate and 0.5% FCS. Cells were then incubated for 24-96 hr. Cell viability, as indicated by reduction of a tetrazolium salt (MTT) to a purple formazan product, was assessed using the CellTiter 96™ kit (Promega, Madison, WI) according to the manufacturer's instructions. Standard curves were constructed to ensure that optical density measurements were within a linear range and to allow optical density readings to be converted to cell number. To assess the pertussis toxin sensitivity of prosaposin receptor agonist effects, cells were incubated in 10 ng/ml pertussis toxin for 24 hr; TNFα in the presence or absence of prosaposin or prosaptide was then added and cell viability assessed at 48 hr. This regimen of pertussis toxin treatment has previously been shown to inhibit prosaptide-induced ERK phosphorylation in iSC cells by Campana (unpublished observation) and thrombin-induced proliferation of CCL39 cells by Chambard et al. (1987).

For trypan blue studies, cells were seeded at $5x10^4$/well in 6-well plates in complete media and grown overnight. Treatments were then added in DMEM containing penicillin, streptomycin, sodium pyruvate and 0.5% FCS and cells grown for a further 48 hr. Cells were stained with trypan blue and viable (unstained) and non-viable cells (stained blue) were scored. Duplicate wells were prepared for each treatment and within each well two groups of 100 cells were scored.

Proliferation of NS20Y cells, as indicated by BrdU incorporation, was measured using the Cell Proliferation ELISA, BrdU colorimetric kit from Boehringer-Mannheim (Indianapolis, IN) according to the manufacturers directions. Cells were seeded as for MTT assays. Prosaptide was added to media containing 0.5% FCS or FCS was added to serum-free media in 2-fold dilution series. Cells were then incubated for 24 - 96 hr.

All experiments were performed in duplicate or triplicate and in each FIG. 6-9, the mean ± S.E.M. of a representative experiment is presented; $n \geq 2$. Pooled data were analyzed using one-way ANOVA and the source of significance ($p<0.05$) was determined using Scheffe's posthoc analysis.

Results. Treatment of NS20Y cells with TNFα resulted in a loss of viability as demonstrated by a decrease in MTT reduction. The effect of TNFα was dose dependent with maximal diminuition at 100 ng/ml TNFα. No loss of viability was seen at 24 hr. Thus, TNFα was administered at 100 ng/ml for 48 or more hours.

FIG. 6A shows that cultures which received TNFα for 48 hr demonstrated a 35% reduction in the number of viable cells as compared to controls. Prosaposin, applied to cells as a single dose at t = 0 hr, partially prevented the loss of viability in a dose dependent manner with greatest protection seen at 5 nM. Similarly, prosaptide administered at t = 0 hr prevented the loss of viability caused by TNFα in a dose-dependent manner. *See,* FIG. 6B. Maximal protection was seen when cells were treated with 50 nM prosaptide with greater than 90% of the cells maintained by 50 nM of the peptide.

A single application of prosaposin at t = 0 hr prevented loss of viability at all time points studied, however, the potency of the effect decreased dramatically over time. *See,* FIG. 7. At 48 hr untreated cultures were 65% as viable as control cultures whereas cells treated with 5 nM prosaposin were 85% as viable. At time points longer than 48 hr, TNFα continued to result in decreased cell viability and at 96 hr viability was approximately 40% as compared to controls whereas prosaposin treated cells were approximately 54% as viable. In contrast, the protective effect of prosaptide was

maintained at its maximum at all time points. At 72 and 96 hr, prosaptide-treated cells were as viable as controls.

The increase in viability of TNFα-insulted cells treated with prosaposin receptor agonist is due to a decrease in cell death. To show this, trypan blue exclusion and BrdU incorporation experiments were conducted. FIG. 8 shows that 25% of cells treated for 48 hr with 100 ng/ml TNFα were trypan blue positive (dead) as compared to 7% positive in control cultures; the increase in cell death was completely prevented in a dose-dependent manner by prosaptide at a maximal concentration of 1nM. FIG. 9 shows that at 48 hr prosaptide did not induce cell proliferation at any dose. The proliferative capacity of the cells was confirmed by demonstrating a dose-dependent stimulation of proliferation by serum. Additionally, prosaptide did not stimulate proliferation at 24, 72 or 96 hr.

Hiraiwa *et al.* (*Proc. Natl. Acad. Sci. USA 94:* 4778-4781, 1997) have demonstrated that prosaposin receptor agonists stimulate ERK phosphorylation and enhance sulfatide content in Schwann cells (Campana *et al., FASEB J. 12*: 307-314, 1998) demonstrated that both of these prosaposin effects are inhibited by pertussis toxin. To show that the neuroprotective action of prosaposin is mediated by the same mechanism, cells were incubated with pertussis toxin at 10 ng/ml overnight and then treated the cells with TNFα in the presence or absence of prosaposin receptor agonists. FIG. 9 shows that pertussis toxin alone caused a decrease of approximately 6% in viability of the cells. Similarly, there was 12% enhancement of the TNFα-induced viability loss when pertussis toxin was added. Treatment of cultures with prosaposin at 5 nM or prosaptide at 50 nM largely prevented the loss of cell viability caused by TNFα. Addition of pertussis toxin caused a 7% and 10% decrease in the protective effect of prosaposin and TX14(A), respectively.

This EXAMPLE demonstrates that prosaposin and a prosaposin-derived peptide of 14 amino acids, TX14(A), prevented the TNFα-induced death of a neuronal cell line. The neuroprotective action of prosaposin receptor agonists was dose-dependent. At the maximally effective doses the protection was almost complete. Prosaposin was able to protect cells from TNFα-induced death at a 10-fold lower molar concentration

than TX14(A). This is likely due to a difference in the binding affinity of the two ligands for the putative prosaposin receptor. The Kd of prosaposin binding to PC12 cells was 2.5 nM while the Kd of prosaptide (TX14(A)) binding was 18.3nM.

Protection of neuronal cells from TNFα neurotoxicity was achieved by a single dose of prosaposin or prosaptide given together with TNFα; there was no pretreatment and no supplementation of the dose during the experiment. The magnitude of protection by prosaposin was greatly reduced at 96 hr as compared to that seen at 48 hr. However, prosaptide maintained 100% protective capacity as long as 96 hr; the longest time point examined. This difference in efficacy may be due to a difference in stability of the two compounds. Prosaposin has been reported by Hiraiwa *et al.* (*Proc. Natl. Acad. Sci. USA 94:* 4778-4781, 1997) to be rapidly cleaved by cathepsin D. Bn contrast, in 50% human serum prosaptide has a half life of greater than 24 hr.

Using trypan blue exclusion and BrdU incorporation assays, the TNFα-induced loss of viability seen using the MTT assay was confirmed to be due to an increase in cell death and that prosaptide prevented this death. A complete prevention of death was seen at 0.5 nM when using trypan blue. This effective concentration is 100-fold less than that observed in the MTT assay. The reason for this discrepancy is unclear, however, it is possibly due to a difference in the sensitivity of the 2 assays. Jabbar and colleagues (1996) also demonstrated discrepancies between results obtained with MTT and results obtained by cell counting. They showed that MTT underestimated the growth inhibition of COR-L23 cells by IFNγ. Similarly, an underestimation of the amount of TNFα induced death was observed when using the MTT assay. There was a 50% reduction in cell viability when the MTT assay was used, whereas the trypan blue assay revealed a 3-fold increase in the number of dead cells when cultures were treated with TNFα. This underestimation may cause a masking of the protective effects of prosaposin receptor agonists at lower concentrations and hence explain the difference between the results obtained using the two assays. The reduction of MTT is not due simply to mitochondrial reductases. While the assay does effectively measure cell viability, the mechanism by which it does so makes it vulnerable to many influences and this may explain discrepancies between the MTT assay and other viability assays.

Despite the discrepancy, TNFα induced cell death and that prosaposin receptor agonists prevented that death.

Prosaposin receptor agonists can stimulate tyrosine phosphorylation in NS20Y cells, iSC cells and primary Schwann cells. In primary Schwann cells, prosaptide-induced ERK phosphorylation is inhibited by pertussis toxin suggesting that the putative prosaposin receptor is linked to a heterotrimeric G-protein containing G_{oa} or G_{ia} subunits. Hiraiwa *et al.* (*Proc. Natl. Acad. Sci. USA 94*: 4778-4781, 1997) have recently presented data to suggest that the association is with $G_{0\alpha}$. Prosaposin receptor agonist-induced enhancement of sulfatide levels has been demonstrated in Schwann cells (*See.*Campana *et al.*, *FASEB J. 12*: 307-314, 1998) and neurite outgrowth in NS20Y cells (*See*, Misasi *et al.*, 1998).

Campana *et al.* (*FASEB J. 12*: 307-314, 1998) have shown that prosaptide stimulates the phosphorylation of PI3-kinase in Schwann cells. PI3-kinase is known to play an integral role in the prevention of neuronal cell death by neurotrophic factors including BDNF, IGF, and NGF and the prevention of death of other cell types.

TNFα can be neurotoxic. This multifunctional cytokine can also be neuroprotective. Whether TNFα acts in a protective or toxic capacity may well be determined by which neuronal cell line is being studied or the regimen of TNFα treatment used. Under similar conditions NS20Y, PC12 and SK-N-MC cells are susceptible to the cytotoxic effects of TNFα, whereas SH-SY5Y and Neuro2A cultures do not lose viability when treated with 100 ng/ml TNFα for up to 96 hr. Furthermore, the susceptibility of SK-N-MC cells to TNFα changes with their differentiation state. When they are differentiated they display a TNFα-induced loss of viability within 48 hr whereas when they are undifferentiated there is no apparent loss of viability until 96 hr.

Prosaposin receptor agonists prevent the TNFα-induced cell death of a neuronal cell. This will have important therapeutic benefits in the treatment of neurodegeneration.

EXAMPLE 2

PROSAPOSIN RECEPTOR AGONISTS INHIBITS JNK2 PHOSPHORYLATION IN SCHWANN CELLS

This EXAMPLE shows that prosaposin receptor agonist TX14(A) inhibits JNK2 phosphorylation in Schwann cells after a 5 minute treatment (*see*, FIG. 10). Additionally, prosaposin receptor agonist TX14(A) enhances Schwann cell production of p100^{PARP} after one hour in low serum media (*see*, FIG. 11).

EXAMPLE 3

PROSAPTIDE ACTIVATES THE MAPK PATHWAY BY A G-PROTEIN-DEPENDENT MECHANISM ESSENTIAL FOR ENHANCED SULFATIDE SYNTHESIS BY SCHWANN CELLS

This EXAMPLE shows that treatment of primary Schwann cells and an immortalized Schwann cell line, iSC, with a 14-mer prosaptide, TX14(A), (10 nM) enhanced phosphorylation of mitogen-activated kinases, ERK1 (p44^{MAPK}; extracellular signal-regulated kinase 1) and ERK2 (p42^{MAPK}; extracellular signal-regulated kinase 2) within 5 minutes that was blocked by 4 hour pretreatment with pertussis toxin. Furthermore, incubation of Schwann cells with the non-hydrolyzable GDP analog, GDP-βS, inhibited TX14(A)-induced ERK phosphorylation. TX14(A) enhanced the sulfatide content of primary Schwann cells 2.5-fold which was inhibited by pretreatment with pertussis toxin or the synthetic MEK inhibitor, PD098059. In addition, TX14(A) increased the tyrosine phosphorylation of all 3 isoforms of the adapter molecule, Shc, which coincided with the association of p60^{Src} and PI 3-kinase. Inhibition of PI 3-kinase by wortmannin blocked TX14(A)-induced ERK phosphorylation. This EXAMPLE demonstrates that TX14(A) uses a pertussis toxin sensitive G-protein pathway to activate ERKs that is essential for enhanced sulfatide synthesis in Schwann cells.

Materials and Methods. TX14(A) (SEQ ID NO:7) was synthesized commercially to 98% purity (AnaSpec, San Jose, CA). Platelet Derived Growth Factor

(PDGF) was purchased from Genzyme (Cambridge, MA). GDP-βS, PD098059, wortmannin (WT) and pertussis toxin (PT) were purchased from CalBiochem (San Diego, CA). Anti-phosphotyrosine monoclonal Ab, anti-Src monoclonal Ab, anti-PI 3-kinase polyclonal Ab and anti-Shc polyclonal Ab were purchased from Upstate Biotechnology Incorporated (Lake Placid, New York).

Two Schwann cell cultures were used; (1) a spontaneously transformed cell line, iSC, from rat primary Schwann cells, as described by Bolin *et al.* (*J. Neurosci. Res. 33:* 231-238, 1992), and primary Schwann cells that were prepared from neonatal rats as described by Assouline *et al.* (In *A Dissection and Tissue Culture Manual of the Nervous System* (Shahar *et al.*, eds), Wiley-Liss, New York, 1989) pp. 247-250. At the first passage, Schwann cells were further selected from fibroblasts using an anti-fibronectin antibody and rabbit complement. This resulted in approximately 99% pure Schwann cell cultures as assessed by S100 and fibronectin immunoflourescence. iSC cells were maintained in DME/F12 containing 10% horse serum and P/S (100 U/mL penicillin and 100 μg/mL streptomycin). Primary Schwann cells were maintained in DMEM containing 10% fetal bovine serum (FBS), P/S, 21 μg/mL bovine pituitary extract and 4 mM forskolin. All cells were incubated at 37°C under humidified 7.5% CO_2.

Primary Schwann cells and iSC cells were grown to 85% confluency in maintenance media and changed to serum free media (SFM) 6 hours (primary Schwann cells) or 16-18 hours (iSC cells) before experimentation. Experiments involving the non-hydrolyzable GDP analogue, GDP-βS, were performed by permeabilizing serum starved cells with saponin (20 μg/mL) for 3 minutes in the presence of GDP-βS. Cells were then rinsed twice with SFM and reincubated at 37°C with GDP-βS for 20 minutes prior to the addition of effectors. Cells were pretreated with either pertussis toxin, PD098059 or wortmannin. In all experiments, cells were stimulated with effectors for 5 minutes, washed 3 times with ice cold PBS containing 1mM sodium vanadate and lysed on ice in lysis buffer as previously described by Campana *et al.* (*Biochem. Biophys. Res. Commun 229:* 706-712, 1996). Protein content of each sample was determined using the bicinchonic acid method (Sigma Chemical Co., St Louis, MO). Western immunoblotting and densitometry were performed as described by Campana *et al.* (*Biochem. Biophys.*

Res. Commun 229: 706-712, 1996), except that nitrocellulose membranes were used instead of PVDF membranes. Differences in treatments were analyzed by ANOVA and treatment means were analyzed by the Student's-Newman-Keuls Multiple Comparisons Test.

ERK activity was assessed using a MAP kinase activity kit (New England Biolabs, Cambridge, MA) with minor modifications. Briefly, Schwann cells were prepared as described above, stimulated with effectors for 5 minutes and lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1 mM sodium vanadate, 1 μg/mL leupeptin and 1 mM PMSF. Protein content of each sample was determined as above. Primary Schwann cell lysates (100 μg) and iSC cell lysates (200 μg) were incubated with 1:200 phospho-MAP kinase antibody overnight at 4°C. Immunoprecipitates were obtained by adding 20 μl (50% slurry) protein A sepharose CL-4B (Sigma, St Louis, MO) and incubating at 4°C for 4 hours or overnight. Beads were washed twice in lysis buffer and subsequently washed in kinase buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM sodium vanadate and 10 mM $MgCl_2$. Immunoprecipitates were incubated at 30°C for 30 minutes in kinase buffer containing 1 μg ELK-1 fusion protein and 100 μM ATP. Reactions were terminated by the addition of 25 μl 3X SDS sample buffer. Samples were boiled for 5 minutes and proteins were resolved by SDS-PAGE). Proteins were electroblotted onto nitrocellulose membrane and ERK activity was identified by immunoblotting with a phospho-ELK-1 antibody followed by detection with ECL (Amersham, Arlington Heights, IL).

iSC cells (approximately 2.0 x 10^7) were incubated in DMEM/F12 without serum 18 hours prior to stimulation with TX14(A) for 5 minutes at 37°C. Cells were then lysed and immunoprecipitated as previously described by Lanfrancone *et al.* (*Oncogene 10:* 907-917, 1995). Protein concentrations were determined by bicinchoninic acid method (Sigma Chemical Co., St Louis, MO). Immunoprecipitates containing equal amounts of protein were resolved by SDS-PAGE and electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). After blocking with 3% BSA and 0.05% Tween 20, membranes were probed with specific antibodies at 4°C

overnight in 1% BSA diluted in T-TBS (20 mM Tris-HCL pH 7.6 150 mM NACl and 0.05% Tween 20). After extensive washing, proteins from the immunocomplexes were detected by horseradish-peroxidase conjugated species specific secondary antibodies (Bio-Rad, Hercules, CA) followed by ECL (Amersham, Arlington Heights, IL).

Primary Schwann cells were incubated in DMEM containing 0.5% FBS with and without effectors for 48 hours. Cells that were treated with pertussis toxin (50 ng/mL) were preincubated for 4 hours in 0.5% FBS containing media before the addition of effectors. Cells treated with the synthetic inhibitor of MEK, PD098059, were preincubated at 37°C for 30 minutes prior to the addition of effectors. Cells were rinsed with PBS, harvested and sonicated in 100 μl distilled water. An aliquot of cell lysate was removed for protein analysis and the remainder was extracted with 5 mL of chloroform/methanol, 2:1 (v/v). Schwann cell lipid extracts were chromatographed and immunostained with an anti-sulfatide monoclonal antibody that is highly specific for sulfatide as described by Hiraiwa *et al.* (*Proc. Natl. Acad. Sci. USA 94:* 4778-4781, 1997). The effect of treatment changes in sulfatide synthesis were tested by comparing the differences by ANOVA and treatment means by the Student's-Newman-Keuls Multiple Comparisons Test.

Results. TX14(A) increased both ERK1 and ERK2 phosphorylation in Schwann cells. There was a larger increase in the ratio of ERK1 phosphorylation to total ERK1 protein (18-fold that of controls) than that of ERK2 (3-fold greater than controls). When iSC cells were preincubated with pertussis toxin, which catalyzes the ADP-ribosylation of $G_i/G_{0\alpha}$ subunits of G-proteins, TX14(A)-induced ERK phosphorylation was inhibited. Similar results were also observed in primary Schwann cells. By contrast, PDGF, which binds to a tyrosine kinase receptor and stimulates proliferation of Schwann cells, stimulated ERK1 (4-6 fold) and ERK2 (2 fold) phosphorylation but was not inhibited by pertussis toxin pretreatment. To further confirm that ERK phosphorylation by TX14(A) involved G-proteins, the iSC cells were incubated with GDP-βS. This treatment also blocked TX14(A)-induced ERK phosphorylation.

ERK protein kinases are activated by phosphorylation of tyrosine and threonine residues and both are required for full protein kinase activity. Because the

antibody used only recognized the phosphorylated tyrosine residue on ERKs, the TX14(A)-induced phosphorylation of ERK was correlated with ERK catalytic activity. Kinase activity was also increased in both primary Schwann cells and iSC cells after treatment with PDGF and TX14(A).

The activation of the adapter protein, Shc, was also examined in TX14(A) signaling. iSC cells expressed all 3 isoforms: $p46^{Shc}$, $p52^{Shc}$, and $p66^{Shc}$. Immunoprecipitation of iSC cell lysates with a polyclonal antibody to all 3 isoforms of Shc, followed by Western blotting with an anti-phosphotyrosine antibody, demonstrated that TX14(A) greatly enhanced tyrosine phosphorylation of all 3 Shc isoforms. Furthermore, 2 unidentified tyrosine phosphorylated proteins were observed in the Shc immunoprecipitates of approximately 60 kDa and 85 kDa in size. Western blotting of Shc immunoprecipitates with an antibody to $p60^{Src}$ revealed that the 60 kDa tyrosine phosphorylated protein was $p60^{Src}$ and an antibody to $p85^{PI\ 3\text{-}kinase}$ revealed that the 85 kDa phosphorylated protein was indeed the p85 subunit of PI 3-kinase. Moreover, after TX14(A) treatment there was more PI3K associated with Shc immunoprecipitates than controls. Blots were reprobed with anti-Shc to demonstrate that equal amounts of unphosphorylated Shc proteins were loaded onto the gel. Subsequently, iSC cell lysates were immunoprecipitated with an antibody to $p60^{Src}$ and Western blotted with an antibody to phosphotyrosine; this showed enhanced tyrosine phosphorylation of PI 3-kinase after treatment with TX14(A). Furthermore, preincubation of iSC cells with wortmannin completely blocked TX14(A)-induced ERK phosphorylation. In unstimulated cells, wortmannin treatment reduced ERK phosphorylation below control levels.

TX14(A) stimulates synthesis of sulfatide in Schwann cells. To determine whether G-protein mediated ERK phosphorylation was involved in synthesis of sulfatide, primary Schwann cells were preincubated with either pertussis toxin or the synthetic inhibitor of MEK, PD098059, before TX14(A) stimulation. The anti-sulfatide monoclonal antibody identified only sulfatide that had the same mobility as purified sulfatide in all samples. In addition, TX14(A) treatment increased the sulfatide content 2.5-fold over controls. Pretreatment with either pertussis toxin or PD098059 inhibited

TX14(A)-induced sulfatide synthesis. The viability of Schwann cells treated with either PD098059 or pertussis toxin after 48 hours did not differ from controls as determined by trypan blue exclusion.

To confirm that the dose of PD098059 used to inhibit sulfatide synthesis also inhibited ERK phosphorylation in primary Schwann cells, ERK phosphorylation experiments in cells pretreated with PD098059 were performed. TX14(A) increased the phosphorylation of ERKs, however, the magnitude of the increase was less than what was observed in iSC cells. The same dose of PD098059 (50 μM) used in the sulfatide experiments blocked TX14(A)-induced phosphorylation of ERK in primary Schwann cells. In addition PD098059 decreased ERK1 and ERK2 phosphorylation below control levels. Timecourse experiments of TX14(A)-induced phosphorylation of ERKs in iSC cells demonstrated that TX14(A) rapidly activates ERK1 and ERK2 within 5 minutes and returned to baseline levels by 30 minutes.

Identification of a G-protein dependent mechanism for TX14(A) signaling. TX14(A) dose-dependently stimulates ERK phosphorylation in both iSC and primary Schwann cells. After quantification and expression of the data as a ratio of phosphorylated ERKs to total ERK proteins, TX14(A) preferentially phosphorylated ERK1, although Schwann cells contained a greater amount of immunoreactive ERK2 protein. The same phenomenon has been observed in PC12 cells and ERK1 is preferentially activated in oligodendrocytes. In this EXAMPLE, TX14(A)-stimulated ERK phosphorylation was blocked by pertussis toxin treatment which indicated that the primary mechanism of activation involved one or more pertussis toxin sensitive G proteins such as G_i or G_0, both of which are abundantly expressed in Schwann cells.

ERK activation is associated with pertussis toxin-sensitive G-protein signaling in COS-7 cells, CHO cells) and Swiss 3T3 cells. The mechanism of MAP kinase activation by G-coupled receptors involves Gβ subunits. Both prosaptides and prosaposin specifically bind to PC12 cells in a dose dependent saturatable manner with high affinity (Kd=2.5 nM and 18 nM, respectively)). Similarly, cell surface binding assays using radio-labeled TX14(A) gave a single high affinity constant for binding to iSC cells with a Kd of 10 nM. These findings demonstrate that prosaposin and TX14(A)

bind to a putative receptor which associates with pertussis toxin-sensitive G-protein to mediate signal transduction. Pertussis toxin-sensitive ERK signaling is known for the insulin-like growth factor receptor tyrosine kinase, as well as the more common 7 transmembrane G-protein coupled receptors.

The pathways of signal transduction which underlie myelination have not been clearly defined. In oligodendrocytes, the initial stages of myelination involves non-receptor tyrosine kinases of the Src family and ERK activation play an important role in process extension. In the peripheral nerve, tissue concentrations of ERKs have been shown to increase after peripheral nerve injury (day 3). ERKs have been localized to activated Schwann cells and increased concomitant with remyelination.

This EXAMPLE demonstrates that inhibition of MEK by PD098059 completely blocked TX14(A)-enhanced synthesis of sulfatide, an essential myelin lipid component of both central and peripheral nervous system myelin, in Schwann cells. This concentration of PD098059 (50 μM) specifically inhibits MEK and not other kinases such as PKC, PI 3-kinase or p38 MAP kinase. PD098059 did decrease ERK phosphorylation below controls suggesting that primary Schwann cells in culture contain autocrine regulated ERKs.

In addition, the timecourse of ERK activation by TX14(A) showed that only 5 minutes of stimulation is sufficient for enhanced sulfatide synthesis observed 48 hours later. Transient activation of ERKs in PC12 cells with growth factors, such as EGF does not lead to pronounced nuclear translocation, so that in Schwann cells, TX14(A)-induced ERK acts in the cytosol to contribute to myelin lipid synthesis. Thus, signal transduction through the ERK pathway is an essential signaling pathway responsible for myelination by Schwann cells.

TX14(A) signaling involved the adapter protein, Shc, and the non-receptor tyrosine kinase, $p60^{Src}$. This EXAMPLE demonstrates that Shc associated with $p60^{Src}$ following TX14(A) stimulation, which coincided with increased tyrosine phosphorylation of Shc. The association of $p60^{Src}$ and Shc has been observed previously in COS-7 cells after lysophosphatidate (LPA) stimulation and has been proposed to be

involved in early activation of ERKs by pertussis toxin-sensitive G-protein coupled receptors.

The results of this EXAMPLE with the MEK inhibitor, PD098059, showed that ERK activation by TX14(A) is due to the $p21^{Ras}$ mediated signaling cascade in Schwann cells. However,this EXAMPLE also demonstrates that PI 3-kinase plays a role in ERK activation in response to TX14(A) based on the ability of wortmannin to block TX14(A)-induced ERK phosphorylation and the observation that TX14(A) induced a larger amount of $p85^{PI\ 3\text{-kinase}}$ in Shc immunoprecipitates coincident with Shc tyrosine phosphorylation. The concentration of wortmannin used in this EXAMPLE has been shown previously to specifically inhibit PI 3-kinase activity in Swiss 3T3 fibroblasts and L6 rat myoblasts. PI 3-kinase has been shown to activate ERKs by a $p21^{Ras}$-independent mechanism and by linkage with G-protein coupled receptors showing that TX14(A) signaling involves multiple and perhaps novel pathways leading to ERK activation.

TX14(A) Role in Myelination. Prosaposin is not only a neurotrophic factor, but an essential factor for events involved in myelination, including prevention of Schwann cell and oligodendrocyte death and synthesis of a myelin lipid, sulfatide. Moreover, prosaposin-deficient transgenic mice have severe hypomyelination in both the central and peripheral nervous system which was apparently due to failure of myelin synthesis, rather than demyelination. The deficiency of myelin in these animals and in prosaposin deficient humans is due to the lack of a myelinotropic effect of prosaposin during development. This EXAMPLE shows that TX14(A), encompassing the neurotrophic region of prosaposin, appeared to exert its trophic effect by binding to a high affinity receptor which activated a pertussis toxin-sensitive G-protein and signaled through ERKs to up regulate the synthesis of sulfatide in Schwann cells. Inhibition of ERK activation blocked enhanced synthesis of sulfatide implicating ERKs as a key signaling component in myelin lipid synthesis.

EXAMPLE 4

EFFECT OF PROSAPOSIN AND TX14(A) ON PROINFLAMMATORY CYTOKINE-INDUCED OLIGODENDROCYTE CELL DEATH

This EXAMPLE demonstrates that prosaposin or the prosaposin-derived peptide TX14(A) (SEQ ID NO:7), can inhibit proinflammatory cytokine-induced apoptosis. Undifferentiated CG4 oligodendrocytes were grown in DMEM containing 10% fetal calf serum. Cells were removed with trypsin and plated in 30 mm petri dishes onto glass coverslips in 0.5% fetal bovine serum for 2 days in the presence of absence of the following effectors: 200 ng/ml TNFα alone or in the presence of 1 nM prosaposin, 5 nM prosaposin, 10 nM TX14(A) or 50 nM TX14(A). The same experiment was also performed using 200 ng/ml IFNγ alone or in the presence of 1 nM prosaposin, 5 nM prosaposin, 10 nM TX14(A) or 50 nM TX14(A). The MTT cell death assay was then performed using a kit (Promega, Madison, WI). This assay measures the MTT dye reduced by mitochondria. In the presence of TNFα and IFNγ, the MTT absorbance decreases due to increased cell death and greater reduction of the MTT dye by mitochondria that are released from lysed cells.

As shown in FIG. 13A, TNFα-induced apoptosis is completely reversed by prosaposin (1 nM and 5 nM) and TX14(A) (10 nM and 50 nM). An inhibitory effect was also observed with IFNγ, albeit not as strong as that obtained with TNFα. *See,* FIG. 13B. Therefore, prosaposin and TX14(A) inhibit TNFα and IFNγ-induced apoptosis in oligodendrocytes.

EXAMPLE 5

L6 MYOBLAST RESCUE

This EXAMPLE demonstrates that TX14(A) inhibits proinflammatory cytokine TNFα-induced apoptosis in L6 myoblasts. L6 myoblasts cells were incubated for 96 hours either in media (control); media with 10 ng/ml TNFα (TNF Category); or

media with 10 ng/ml TNFα and 200 ng/ml TX14(A). *See,* FIG. 14. Cell death was measured by trypan blue assay as described in EXAMPLE 3. Cell death was inhibited in L6 myoblasts incubated with TNFα and TX14(A), as compared to L6 myoblasts incubated with TNFα only (approximately 60% cell death). Therefore, prosaposin receptor agonist treatment inhibits TNFα-induced apoptosis in myoblasts.

EXAMPLE 6

EFFECT OF TX14(A) ON THERMAL HYPERALGESIA FOLLOWING ENDONEURIAL INJECTION OF TNFα

This EXAMPLE demonstrates that a prosaposin-derived peptide, TX14(A) peptide (prosaptide; SEQ ID NO:7), was effective in treating TNFα-induced inflammation. The inflammatory component of peripheral nerve injury may affect the development of local neuropathologic changes as well as the onset of hyperalgesia, the characteristic features of experimental neuropathic pain states. *See,* Wagner *et al.* (*NeuroReport* 7: 2897-2901, 1996).

TNFα (2.5 pg/ml) was injected directly into the endoneurial space of normal rat nerves. In a parallel experiment, TX14(A) (200 μg/kg) was injected subcutaneously prior to injection of TNF-α. The resulting effects on behavior were monitored for 1 week. Behavioral measurements assessed the response to thermal stimuli and were expressed as a difference score (ipsilateral minus contralateral paw).

As shown in FIG. 15, the TX14(A) peptide dramatically reduced TNFα-induced thermal hyperalgesia in the rat model. Therefore, TX14(A) peptide (prosaptide) inhibits TNFα-induced neuropathic pain in the endoneurial space of normal rat nerves.

EXAMPLE 7

IN VIVO UPTAKE OF PROSAPOSIN-DERIVED PEPTIDES BY THE CENTRAL NERVOUS SYSTEM

This EXAMPLE demonstrates that prosaposin-derived peptides cross the blood-brain barrier.

An octadecamer (SEQ ID NO:5) consisting of amino acids 12-29 of saposin C with a tyrosine substituted for valine at position 12 was chemically synthesized on an Applied Biosystems Model 430 peptide synthesizer. The peptide was then radioiodinated by the lactoperoxidase method; 20 x 10^6 cpm radiolabeled peptide were injected into the auricles of rats. The animals were sacrificed after 1 hr and 24 hr, and the hearts were perfused with isotonic saline in order to remove the blood from the brain.

In order to determine the percentage of peptide uptake, the brain was then counted in a gamma counter. In addition, the brain was homogenized and fractionated into a capillary rich fraction (pellet) and a parenchymal brain fraction (supernatant) after dextran centrifugation. *See,* Triguero *et al.* (*J. Neurochem., 54:*1882-1888, 1990). This method allows for the discrimination between radiolabeled peptide within blood vessels and that within the brain. After 24 hr, 0.017% of the injected peptide (SEQ ID NO:5) was detected in whole brain; 75% of the label was in the parenchymal fraction and 25% was in the capillary fraction. At 1 hr, 0.03% of the injected dose was present in whole brain.

The prosaposin-derived peptide SEQ ID NO:7 also was assayed for ability to cross the blood-brain barrier as follows. A female Sprague-Dawley rat was anesthesized with methoxyflurane, and approximately 20 µg peptide SEQ ID NO:2 (3.2 x 10^8 cpm) was injected into the tail vein. After 40 minutes, the rat was sacrificed by ether anesthesia and perfused with about 250 ml PBS through the heart. The total amount of peptide in brain, liver and blood was calculated as a percentage of the injected material as shown in TABLE 3. In order to determine the localization in brain, the capillary depletion method of Triguero (*J. Neurochem. 54:*1882, 1990) was used to separate brain tissue into a parenchyma fraction and a brain capillary fraction. The fractionation results showed that 87% of the SEQ ID NO:7 peptide present in brain was localized to brain parenchyma while 13% was found in brain capillary.

TABLE 3			
TISSUE	WEIGHT	TOTAL CPM IN TISSUE	PERCENTAGE OF INITIAL CPM
Brain	1.3 g	161,000	0.050
Liver	8.8 g	5.2×10^6	1.625
Blood	about 22 µl	1.01×10^8	31.6

In a similar experiment in which rats were sacrificed after 3 hr treatment with SEQ ID NO:7, 0.06% of the peptide was evident in brain, of which 85% was in the parenchyma. These results demonstrate that at least some of the prosaposin-derived peptide SEQ ID NO:7 crossed the blood brain barrier and was concentrated in the brain parenchyma rather than the vascular endothelium (blood vessels). The percentage of peptide that crossed the blood brain barrier is in the mid-range of peptides that cross the barrier as set forth in Banks, *supra*.

In order to determine the percentage of intact material in the brain, liver and blood, radiolabeled material (SEQ ID NO:7) isolated from the tissues was analyzed by high pressure liquid chromatography. To normalize for degradation during processing of tissue homogenates, peptide SEQ ID NO:7 was added to tissue homogenates. The extent of degradation observed with the added peptide material was used to normalize for degradation during tissue processing. After normalization, the results were as follows: SEQ ID NO:2 was about 60% intact in brain; about 80% intact in liver and about 40% intact in blood. In a second experiment, peptide SEQ ID NO:7 was about 68% intact in brain. These results indicate that the peptide SEQ ID NO:7 crosses the blood brain barrier and is largely intact in brain.

EXAMPLE 8

ISOLATION OF PROSAPOSIN RECEPTOR FROM WHOLE RAT BRAIN

A 54 kDa protein has been identified as the receptor for prosaposin as described in this EXAMPLE: A prosaposin receptor protein was isolated from whole rat brain, rat cerebellum and mouse neuroblastoma cells using the plasma membrane P-100 fraction. Briefly, cells or tissues were solubilized and centrifuged at 14,000 rpm to remove debris. The supernatant was centrifuged at 40,000 rpm for 1 hr at 4°C. The pellet, enriched in plasma membrane, was solubilized in RIPA buffer (10 mM MOPS, pH 7.5, 0.3 M sucrose, 5 mM EDTA, 1% Trasylol, 10 μM leupeptin and 10 μM pepstatin). This P-100 fraction was applied to an affinity column containing the bound, active 14-mer fragment of saposin C, TX14(A). The column was washed with 0.05 M NaCl to elute loosely-bound proteins followed by 0.25 M NaCl that eluted the putative 54 kDa prosaposin receptor. In addition, it was determined that the 54-60 kDa protein could be eluted using a 100-fold excess of unbound peptide thus demonstrating specific elution. The 54 kDa protein was approximately 90% pure as judged by SDS-PAGE. The protein was purified to homogeneity using HPLC and eluted at 50% acetonitrile in an acetonitrile/water gradient on a Vydac C4 column. After treatment with the cross-linking reagent disuccinimidyl suberate (DSS; Pierce, Rockford, IL), the 54 kDa protein bound irreversibly to ^{125}I labeled saposin C as evidenced by the 66 kDa molecular weight of the complex (54 kDa + 12 kDa).

EXAMPLE 9

ISOLATION OF PROSAPOSIN RECEPTOR; EVIDENCE FOR A G-PROTEIN ASSOCIATED RECEPTOR

In this EXAMPLE, the prosaposin receptor was partially purified from baboon brain membranes by affinity chromatography using a saposin C-column. The purified preparation gave a single major protein band with an apparent molecular weight

of 54 kDa on SDS-PAGE. Affinity cross-linking of 11 kDa ^{125}I-saposin C demonstrated the presence of a 66 kDa product, indicative of an apparent molecular weight of 55 kDa for the receptor. A GTPγS-binding assay using cell membranes from SHSY5Y neural cells demonstrated agonist stimulated binding of [^{35}S]GTPγS upon treatment with prosaptide TX14(A) a peptide from the neurotrophic region; maximal binding was obtained at 2 nM. TX14(A) stimulated binding was abolished by prior treatment of SHSY5Y cells with pertussis toxin and by a scrambled and an all D-amino acid-derivative of the 14-mer. A 14-mer mutant prosaptide competed with TX14(A) with a Ki of 0.7 nM. Immunoblot analysis using an antibody against the $G_{0\alpha}$ subunit demonstrated that the purified receptor preparation contained a 40 kDa reactive band consistent with association of $G_{0\alpha}$ and the receptor. The results of this EXAMPLE show that the signaling induced by prosaposin and TX14(A) is generated by binding to a G_0-protein associated receptor.

TX14(A) also bound to PC12 cells and iSC Schwann cells with Kd values of 18.3 nM and 10 nM and increased phosphorylation of MAP kinase (15,16). These findings suggested the presence of a specific receptor for prosaposin which triggers a MAP kinase cascade. In this EXAMPLE, a prosaposin receptor is characterized from baboon brain membranes and SHSY5Y cells as a G-protein associated receptor.

Materials and Methods. Baboon brains were frozen in liquid nitrogen immediatelly after death and stored at -70°C until use. Chemically synthesized peptides including TX14(A) (SEQ ID NO:7) and a 14-mer mutant prosaptide, 14M1, with a single amino acid substitution with aspartic acid replacing asparagine 6 in TX14(A) were from AnaSpec, Inc. (San Jose, CA), and were more than 97% pure. A saposin C-column was prepared by conjugation of carbohydrate-free chemically synthesized saposin C with Affigel-10 (Bio-Rad, Hercule, CA) by the manufacture's instruction. An antibody against human saposin C was purified by the procedure described by Hiraiwa *et al.* (*Arch. Biochem. Biophys. 341:* 17-24, 1997). Saposin C purified from Gaucher's spleen (19) was labeled with ^{125}I-NaI (New England Nuclear Biolabs. Cambridge, MA) using Iodobeads (Pierce, Rockford, IL) and desalted by Sephadex G-10 column. The labeled saposin C gave a single band of 11 kDa on autoradiography after Tricine/SDS-PAGE

(18). Protease inhibitors were from Sigma Chemical Ltd. (St Louis, MO). Triton X-100 and sodium deoxycholate were purchased from Calbiochem. (La Jolla, CA). A polyclonal antibody specific for the human $G_{0\alpha}$ subunit was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals and reagents were highest grade available.

Affinity purification of the receptor. All purification procedures were carried out at 4°C unless specified. Baboon brains (1.6 kg) from Southwest Research Institute (San Antonio, TX) were washed in chilled 10 mM MOPS, pH 7.5, containing 0.3 M sucrose and a cocktail of protease inhibitors (5 mM EDTA, 1 mM PMSF, and 5 μg/ml of leupeptin, aprotinin, and pepstatin) (Buffer I). The brains were homogenized in a teflon-glass homogenizer in 3 volumes of Buffer I and centrifuged at 1,500 X g for 20 min. The supernatant was then centrifuged at 100,000 X g for 60 min and the pellet was washed with Buffer I. Then, the pellet was suspended in lysis buffer (10 mM Tris-HCl, pH 7.5, containing 1% sodium deoxycholate, 1% Triton X-100, and the same protease inhibitor cocktail as in Buffer I) and incubated for 60 min on ice with shaking. After removal of the insoluble materials by centrifugation, the supernatant was mixed with 20 ml of saposin C-beads and rotated for 12 hr. The beads were packed into a column and washed with 1 mM sodium phosphate buffer, pH 7.5, containing 0.1% Triton X-100 until protein was not detected in the eluate. The proteins bound to the beads were eluted with 0.1 M glycine-HCl buffer, pH 3.0, at room temperature (affinity purified preparation).

Affinity cross-linking. The affinity purified preparation (4 μg of protein) was dialyzed against 4 changes of 1 liter of 1 mM sodium phosphate buffer, pH 7.5, containing 0.1% Triton X-100 and then concentrated to 1.4 ml by ultrafiltration using Amicon PM-10 membrane. Cross-linking was performed by incubating the concentrated samples (0.3 μg protein) with ^{125}I-saposin C (12.4 ng, 103 cpm/pg) in a final volume of 200 μl of 0.25 M MOPS, pH 7.5, in the presence or absence of 1,000-fold excess of unlabeled saposin C. After 90 min-incubation at room temperature, 20 μl of 30 mM disuccinimidyl suberate (Pierce, Rockford, IL) was added to the reaction mixtures and incubated for further 20 min. The reaction was terminated by the addition of 12 μl of 1 M Tris-HCl, pH 7.5, and the mixtures were left for 20 min at room temperature. The

product cross-linked with saposin C was immunoprecipitated by an affinity purified anti saposin C antibody (2 μg), recovered by Protein A-insoluble (Sigma) then subjected to SDS-PAGE. After protein staining, the gel was dried and then exposed to a Kodak film, BioMax, at -80°C.

GTPγS-binding. The SHSY5Y assay was essentially carried out as described by Campana *et al.* (*Biochem. Biophys. Res. Commun 229:* 706-712, 1996), using SHSY5Y cell membrane preparations. The reaction was performed by incubation of the membrane preparations (50-100 μg protein) with 125 μCi of [^{35}S]-GTPγS (New England Nuclear Biolabs, 1250 Ci/mmol). In our experiments, a concentration of 3 μM GDP was added to amplify the difference between ligand stimulated and background binding. Unlabelled GTPγS (10 nM) was also added to define non-specific binding and this value was subtracted from specific binding. All assays were performed in duplicate.

Results. A putative prosaposin receptor was partially purified from baboon brain membranes. A solubilized membrane preparation was purified by affinity chromatography using a saposin C-column. From 1.6 kg of baboon brain, about 25 μg of the purified preparation was obtained. The purified preparation gave one major band with a molecular weight of 54 kDa on SDS-PAGE. Similar electrophoretic patterns were also observed in purified preparations from membrane fractions of human brain, pig brain and PC12 cells. Cross-linking experiment using ^{125}I-saposin C and the purified preparation demonstrated the presence of a 66 kDa band. On the other hand, no band was observed in the sample cross-linked in the presence of unlabeled saposin C. Since saposin C has a molecular weight of 11 kDa, a molecular weight of the putative receptor was calculated as 55 kDa.

MAP kinase phosphorylation induced by prosaposin receptor agonists in primary Schwann cells is blocked by treatment with pertussis toxin. To investigate whether prosaptides interacted with a G-protein coupled receptor, a GTPγS-binding assay was performed using TX14(A) and membrane preparations from SHSY5Y cells. As shown in FIG. 13A, agonist-stimulated binding was increased by 50-60% above control values in a dose-dependent manner at a maximal concentration of 2 nM. Activation peaked at 2 nm and was bimodal similar to MAP kinase activation in PC12

cells. Other agonists which were active included saposin C (0.3 nM), and a 14-mer derived from the neurotrophic sequence of rat saposin C (SELIINNATEELLY; SEQ ID NO:12). A mutant peptide 14M1 (TXLIDDNATEEILY; where X=D-alanine) inhibited the stimulation of TX14(A) in a dose-dependent manner with maximal inhibition at a concentration of 0.5 nM. Pretreatment of SHSY5Y cells for 4 hr with pertussis toxin (100 ng/ml) prior to membrane preparation abolished the agonist-stimulated binding of TX14(A). An all D-amino acid-derivative of TX14(A) and a scrambled peptide derivative of TX14(A) were inactive. Furthermore, the purified receptor preparation was analyzed for G-proteins by western blotting using an antibody against $G_{0\alpha}$. The purified preparation contained cross-reacting material of 40 kDa indicating that $G_{0\alpha}$ copurified with the receptor.

This EXAMPLE shows that the prosaposin receptor has a molecular weight of 55 kDa and is a G_0 protein-associated receptor. Saposin C also interacts with a 56 kDa lysosomal protein, glucocerebrosidase, to stimulate the enzymic hydrolysis of glucocerebroside. Western blot analysis using an antibody against purified human glucocerebrosidase gave no cross-reacting material in the purified receptor preparation. Prosaposin receptor agonists induced MAP kinase phosphorylation in Schwann cells, and this phosphorylation was blocked both by the treatment with pertussis toxin and a non-hydrolyzable GDP analog, GDPβS. GTPγS-binding to cell membranes has been utilized to characterize agonist-promoted activation of several G-protein associated receptors including opioid receptors and 5-hydroxy tryptophane receptors. The assay relies upon agonist-promoted GDP/GTP exchange occurring at the G-protein level within the receptor/G-protein complex; [^{35}S]GTPγS binding is used to assess receptor activation since GTPγS is only slowly hydrolyzed by the intrinsic GTPase activity of the G-protein. Using SHSY5Y membrane preparations, agonist stimulated binding by μ-opioid agonists was about twice the control level whereas we obtained about 50-70% augmentation using TX14(A) and saposin C. Stimulation was dose dependent, saturable and inhibited by a mutant peptide. These results demonstrated that saposin C and prosaptides were active as ligands in a functional measure of receptor-associated G-protein activation. In addition

the evidence that $G_{0\alpha}$ copurifies with the putative prosaposin receptor indicated that this pertussis toxin-sensitive G protein was associated with the receptor.

It should be noted that the present invention is not limited to only those embodiments described in the DETAILED DESCRIPTION. Any embodiment that retains the spirit of the present invention should be considered to be within its scope. However, the invention is only limited by the scope of the following claims.

SEQUENCE LISTING

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<120> Inhibition of Apoptosis Using Prosaposin Receptor Agonists

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gaa ata ctc gac gct ttt gac aaa atg tgc tcg aag ctg ccg aag tcc  1056
Glu Ile Leu Asp Ala Phe Asp Lys Met Cys Ser Lys Leu Pro Lys Ser
            340                 345                 350

ctg tcg gaa gag tgc cag gag gtg gtg gac acg tac ggc agc tcc atc  1104
Leu Ser Glu Glu Cys Gln Glu Val Val Asp Thr Tyr Gly Ser Ser Ile
        355                 360                 365

ctg tcc atc ctg ctg gag gag gtc agc cct gag ctg gtg tgc agc atg  1152
Leu Ser Ile Leu Leu Glu Glu Val Ser Pro Glu Leu Val Cys Ser Met
    370                 375                 380

ctg cac ctc tgc tct ggc acg cgg ctg cct gca ctg acc gtt cac gtg  1200
Leu His Leu Cys Ser Gly Thr Arg Leu Pro Ala Leu Thr Val His Val
385                 390                 395                 400

act cag cca aag gac ggt ggc ttc tgc gaa gtg tgc aag aag ctg gtg  1248
Thr Gln Pro Lys Asp Gly Gly Phe Cys Glu Val Cys Lys Lys Leu Val
                405                 410                 415

ggt tat ttg gat cgc aac ctg gag aaa aac agc acc aag cag gag atc  1296
Gly Tyr Leu Asp Arg Asn Leu Glu Lys Asn Ser Thr Lys Gln Glu Ile
            420                 425                 430

ctg gct gct ctt gag aaa ggc tgc agc ttc ctg cca gac cct tac cag  1344
Leu Ala Ala Leu Glu Lys Gly Cys Ser Phe Leu Pro Asp Pro Tyr Gln
        435                 440                 445

aag cag tgt gat cag ttt gtg gca gag tac gag ccc gtg ctg atc gag  1392
Lys Gln Cys Asp Gln Phe Val Ala Glu Tyr Glu Pro Val Leu Ile Glu
    450                 455                 460

atc ctg gtg gag gtg atg gat cct tcc ttc gtg tgc ttg aaa att gga  1440
Ile Leu Val Glu Val Met Asp Pro Ser Phe Val Cys Leu Lys Ile Gly
465                 470                 475                 480

gcc tgc ccc tcg gcc cat aag ccc ttg ttg gga act gag aag tgt ata  1488
Ala Cys Pro Ser Ala His Lys Pro Leu Leu Gly Thr Glu Lys Cys Ile
                485                 490                 495

tgg ggc cca agc tac tgg tgc cag aac aca gag aca gca gcc cag tgc  1536
Trp Gly Pro Ser Tyr Trp Cys Gln Asn Thr Glu Thr Ala Ala Gln Cys
            500                 505                 510

aat gct gtc gag cat tgc aaa cgc cat gtg tgg aac taggaggagg       1582
Asn Ala Val Glu His Cys Lys Arg His Val Trp Asn
        515                 520

aatattccat cttggcagaa accacagcat tggttttttt ctacttgtgt gtctgggggga 1642

atgaacgcac agatctgttt gactttgtta taaaaatagg gctcccccac ctcccccatt 1702

tctgtgtcct ttattgtagc attgctgtct gcaagggagc ccctagcccc tggcagacat 1762

agctgcttca gtgcccctttt tctctctgct agatggatgt tgatgcactg gaggtctttt 1822

agcctgccct tgcatggcgc ctgctggagg aggagagagc tctgctggca tgagccacag 1882
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            20   25   30

Cys Gln Asn Val Lys Thr Ala Ser Asp Cys Gly Ala Val Lys His Cys
        35   40   45

Leu Gln Thr Val Trp Asn Lys Pro Thr Val Lys Ser Leu Pro Cys Asp
    50   55   60

Ile Cys Lys Asp Val Val Thr Ala Ala Gly Asp Met Leu Lys Asp Asn
65   70   75   80

Ala Thr Glu Glu Glu Ile Leu Val Tyr Leu Glu Lys Thr Cys Asp Trp
                85   90   95

Leu Pro Lys Pro Asn Met Ser Ala Ser Cys Lys Glu Ile Val Asp Ser
            100   105   110

Tyr Leu Pro Val Ile Leu Asp Ile Ile Lys Gly Glu Met Ser Arg Pro
115 120 125

Gly Glu Val Cys Ser Ala Leu Asn Leu Cys Glu Ser Leu Gln Lys His
130 135 140

Leu Ala Glu Leu Asn His Gln Lys Gln Leu Glu Ser Asn Lys Ile Pro
145 150 155 160

Glu Leu Asp Met Thr Glu Val Val Ala Pro Phe Met Ala Asn Ile Pro
165 170 175

Leu Leu Leu Tyr Pro Gln Asp Gly Pro Arg Ser Lys Pro Gln Pro Lys
180 185 190

Asp Asn Gly Asp Val Cys Gln Asp Cys Ile Gln Met Val Thr Asp Ile
195 200 205

Gln Thr Ala Val Arg Thr Asn Ser Thr Phe Val Gln Ala Leu Val Glu
210 215 220

His Val Lys Glu Glu Cys Asp Arg Leu Gly Pro Gly Met Ala Asp Ile
225 230 235 240

Cys Lys Asn Tyr Ile Ser Gln Tyr Ser Glu Ile Ala Ile Gln Met Met
245 250 255

Met His Met Gln Pro Lys Glu Ile Cys Ala Leu Val Gly Phe Cys Asp
260 265 270

Glu Val Lys Glu Met Pro Met Gln Thr Leu Val Pro Ala Lys Val Ala
275 280 285

Ser Lys Asn Val Ile Pro Ala Leu Glu Leu Val Glu Pro Ile Lys Lys
290 295 300

His Glu Val Pro Ala Lys Ser Asp Val Tyr Cys Glu Val Cys Glu Phe
305 310 315 320

Leu Val Lys Glu Val Thr Lys Leu Ile Asp Asn Asn Lys Thr Glu Lys
325 330 335

Glu Ile Leu Asp Ala Phe Asp Lys Met Cys Ser Lys Leu Pro Lys Ser
340 345 350

Leu Ser Glu Glu Cys Gln Glu Val Val Asp Thr Tyr Gly Ser Ser Ile
355 360 365

Leu Ser Ile Leu Leu Glu Glu Val Ser Pro Glu Leu Val Cys Ser Met
370 375 380

Leu His Leu Cys Ser Gly Thr Arg Leu Pro Ala Leu Thr Val His Val
385 390 395 400

Thr Gln Pro Lys Asn Gly Gly Phe Cys Glu Val Cys Lys Lys Leu Val
405 410 415

Gly Tyr Leu Asp Arg Asn Leu Glu Lys Asn Ser Thr Lys Gln Glu Ile
420 425 430

Leu Ala Ala Leu Glu Lys Gly Cys Ser Phe Leu Pro Asp Pro Tyr Gln
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Lys Gln Cys Asp Gln Phe Val Ala Glu Tyr Glu Pro Val Leu Ile Glu
450 455 460

Ile Leu Val Glu Val Met Asp Pro Ser Phe Val Cys Leu Lys Ile Gly
465 470 475 480

Ala Cys Pro Ser Ala His Lys Pro Leu Leu Gly Thr Glu Lys Cys Ile
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Glu Val Val Asp Thr Tyr Gly Ser Ser Ile Leu Ser Ile Leu Leu Glu
50 55 60

Glu Val Ser Pro Glu Leu Val Cys Ser Met Leu His Leu Cys Ser Gly
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Ile Leu

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Thr Glu Lys Glu Ile Leu
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<212> PRT

<213> Artificial Sequence

<220>

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TX14A

<220>

<221> VARIANT

<222> (2)

<223> The alanine at position 2 is a D amino acid.

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<213> Mus musculus

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<213> Rattus sp.

<400> 9
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            20
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<210> 10

<211> 22

<212> PRT

<213> Cavia guianae

<400> 10

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Thr Glu Glu Lys Ile Ile
            20
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<213> Bos sp.

<220>

<223> Description of Unknown Organism:bovine

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Thr Glu Glu Glu Ile Leu
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<211> 14

<212> PRT

<213> Rattus sp.

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1 5 10

CLAIMS

What is claimed:

1. A method for inhibiting apoptosis in a cell, comprising contacting the cell with an apoptosis-inhibiting amount of a prosaposin receptor agonist, wherein the prosaposin receptor agonist inhibits apoptosis in the cell.

2. The method of claim 1, wherein the apopotosis is caspase- mediated.

3. The method of claim 2, wherein the apoptosis is induced by a proinflammatory cytokine-induced apoptosis.

4. The method of claim 3, wherein the proinflammatory cytokine is TNFα.

5. The method of claim 3, wherein the proinflammatory cytokine is IFNγ.

6. The method of claim 1, wherein the prosaposin receptor agonist has at least about 11 amino acids and comprises the amino acid sequence LeuIleXaa$_1$AsnAsnXaa$_1$ThrXaa$_2$Xaa$_3$Xaa$_2$Xaa$_1$, wherein:
Xaa$_1$ is any amino acid;
Xaa$_2$ is a charged amino acid; and
Xaa$_3$ is optionally present and, when present, is a charged amino acid.

7. The method of claim 6, wherein the prosaposin receptor agonist comprises a peptide selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12.

8. The method of claim 1, wherein the cell is selected from the group consisting of an oligodendrocyte, neuron, Schwann cell, and myocyte.

9. The method of claim 1, wherein the apoptosis is inhibited *in vitro*.

10. The method of claim 1, wherein the apoptosis is inhibited *in vivo*.

11. The method of claim 1, wherein the apoptosis is associated with a disorder selected from a group consisting of rheumatoid arthritis, Crohn's disease, irritable bowel syndrome, asthma, cardiac infarction, congestive heart failure, multiple sclerosis, acute disseminated inflammatory leukoencephalitis, progressive multifocal leukoencephalitis, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, ischemic heart disease, Guillain-Barre disease, traumatic brain injury, traumatic spinal cord injury, alopecia, AIDS dementia, cerebral malaria, HTLV neuropathy, inflammatory neurodegenerative disease, and toxin-induced liver disease.

12. A method of ameliorating neuropathic pain associated with proinflammatory cytokine, comprising
administering a neuropathic pain-alleviating amount of a prosaposin receptor agonist to a subject suffering from neuropathic pain caused by a proinflammatory cytokine.

13. The method of claim 12, wherein the proinflammatory cytokine is TNFα.

14. The method of claim 12, wherein the proinflammatory cytokine is IFNγ.

15. The method of claim 12, wherein the prosaposin receptor agonist has at least about 11 amino acids and comprises the amino acid sequence LeuIleXaa$_1$AsnAsnXaa$_1$ThrXaa$_2$Xaa$_3$Xaa$_2$Xaa$_1$, wherein:
Xaa$_1$ is any amino acid;
Xaa$_2$ is a charged amino acid; and
Xaa$_3$ is optionally present and, when present, is a charged amino acid.

16. The method of claim 15, wherein the prosaposin receptor agonist comprises a peptide selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12.

FIGURE 1

Prosaposin cDNA (2740) base pair nucleic acid linear cDNA)

SEQ ID NO:1

ATGTACGCCCTCTTCCTCCTGGCCAGCCTCCTGGGCGCGGCTCTAGCCGGCCCGGTCCTTGGACT
GAAAGAATGCACCAGGGGCTCGGCAGTGTGGTGCCAGAATGTGAAGACGGCGTCCGACTGCGGGG
CAGTGAAGCACTGCCTGCAGACCGTTTGGAACAAGCCAACAGTGAAATCCCTTCCCTGCGACATA
TGCAAAGACGTTGTCACCGCAGCTGGTGATATGCTGAAGGACAATGCCACTGAGGAGGAGATCCT
TGTTTACTTGGAGAAGACCTGTGACTGGCTTCCGAAACCGAACATGTCTGCTTCATGCAAGGAGA
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GCAGCTGGAGTCCAATAAGATCCCAGAGCTGGACATGACTGAGGTGGTGGCCCCCTTCATGGCCA
ACATCCCTCTCCTCCTCTACCCTCAGGACGGCCCCCGCAGCAAGCCCCAGCCAAAGGATAATGGG
GACGTTTGCCAGGACTGCATTCAGATGGTGACTGACATCCAGACTGCTGTACGGACCAACTCCAC
CTTTGTCCAGGCCTTGGTGGAACATGTCAAGGAGGAGTGTGACCGCCTGGGCCCTGGCATGGCCG
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CCCAAGGAGATCTGTGCGCTGGTTGGGTTCTGTGATGAGGTGAAAGAGATGCCCATGCAGACTCT
GGTCCCCGCCAAAGTGGCCTCCAAGAATGTCATCCCTGCCCTGGAACTGGTGGAGCCCATTAAGA
AGCACGAGGTCCCAGCAAAGTCTGATGTTTACTGTGAGGTGTGTGAATTCCTGGTGAAGGAGGTG
ACCAAGCTGATTGACAACAACAAGACTGAGAAAGAAATACTCGACGCTTTTGACAAAATGTGCTC
GAAGCTGCCGAAGTCCCTGTCGGAAGAGTGCCAGGAGGTGGTGGACACGTACGGCAGCTCCATCC
TGTCCATCCTGCTGGAGGAGGTCAGCCCTGAGCTGGTGTGCAGCATGCTGCACCTCTGCTCTGGC
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CATGTGTGGAACTAGGAGGAGGAATATTCCATCTTGGCAGAAACCACAGCATTGGTTTTTTTCTA
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CAGACATAGCTGCTTCAGTGCCCCTTTTCTCTCTGCTAGATGGATGTTGATGCACTGGAGGTCTT
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GCACCCCTCCCTTGTCTCATAGATGCTCCTTTTGACCTTTTCAAATAAATATGGATGGCGAGCTC
CTAGGCCTCTGGCTTCCTGGTAGAGGGCGGCATGCCGAAGGGTCTGCTGGGTGTGGATTGGATGC
TGGGGTGTGGGGGTTGGAAGCTGTCTGTGGCCCACTTGGGCACACTTGGGCACCCACGCTTCTGT
CCACTTCTGGTTGCCAGGAGACAGCAAGCAAAGCCAGCAGGACATGAAGTTGCTATTAAATGGAC
TTCGTGATTTTTGTTTTGCACTAAAGTTTCTGTGATTTAACAATAAAATTCTGTTAGCCAGAAAA
AAAAAAAAAAAAAAAAA

FIGURE 2

Prosaposin (523 amino acid N-terminal peptide)

SEQ ID NO:2

MetTyrAlaLeuPheLeuLeuAlaSerLeuLeuGlyAlaAlaLeuAlaGlyProValLeuGly
LeuLysGluCysThrArgGlySerAlaValTrpCysGlnAsnValLysThrAlaSerAspCys
GlyAlaValLysHisCysLeuGlnThrValTrpAsnLysProThrValLysSerLeuProCys
AspIleCysLysAspValValThrAlaAlaGlyAspMetLeuLysAspAsnAlaThrGluGlu
GluIleLeuValTyrLeuGluLysThrCysAspTrpLeuProLysProAsnMetSerAlaSer
CysLysGluIleValAspSerTyrLeuProValIleLeuAspIleIleLysGlyGluMetSer
ArgProGlyGluValCysSerAlaLeuAsnLeuCysGluSerLeuGlnLysHisLeuAlaGlu
LeuAsnHisGlnLysGlnLeuGluSerAsnLysIleProGluLeuAspMetThrGluValVal
AlaProPheMetAlaAsnIleProLeuLeuLeuTyrProGlnAspGlyProArgSerLysPro
GlnProLysAspGlyAspValCysGlnAspCysIleGlnMetValThrAspIleGlnThrAla
ValArgThrAsnSerThrPheValGlnAlaLeuValGluHisValLysGluGluCysAspArg
LeuGlyProGlyMetAlaAspIleCysLysAsnTyrIleSerGlnTyrSerGluIleAlaIle
GlnMetMetMetHisMetGlnProLysGluIleCysAlaLeuValGlyPheCysAspGluVal
LysGluMetProMetGlnThrLeuValProAlaLysValAlaSerLysAsnValIleProAla
LeuAspLeuValAspProIleLysLysHisGluValProAlaLysSerAspValTyrCysGlu
ValCysGluPheLeuValLysGluValThrLysLeuIleAspAsnAsnLysThrGluLysGlu
IleLeuAspAlaPheAspLysMetCysSerLysLeuProLysSerLeuSerGluGluCysGln
GluValValAspThrTyrGlySerSerIleLeuSerIleLeuLeuGluGluValSerProGlu
LeuValCysSerMetLeuHisLeuCysSerGlyThrArgLeuProAlaLeuThrValHisVal
ThrGlnProLysAspGlyGlyPheCysGluValCysLysLysLeuValGlyTyrLeuAspArg
AsnLeuGluLysAsnSerThrLysGlnGluIleLeuAlaAlaLeuGluLysGlyCysSerPhe
LeuProAspProTyrGlnLysGlnCysAspGlnPheValAlaGluTyrGluProValLeuIle
GluIleLeuValGluValMetAspProSerPheValCysLeuLysIleGlyAlaCysProSer
AlaHisLysProLeuLeuGlyTHrGluLysCysIleTrpGlyProSerTyrTrpCysGlnAsn
THrGluThrAlaAlaGlnCysAsnAlaValGluHisCysLysArgHisValTrpAsn

Saposin C

(80 amino acid peptide)

SEQ ID NO:3

SerAspValTyrCysGluValCysGluPheLeuValLysGluValThrLysLeuIleAspAsn
AsnLysThrGluLysGluIleLeuAspAlaPheAspLysMetCysSerLysLeuProLysSer
LeuSerGluGluCysGlnGluValValAspThrTyrGlySerSerIleLeuSerIleLeuLeu
GluGluValSerProGluLeuValCysSerMetLeuHisLeuCysSerGly

FIGURE 3
PROSAPOSIN-DERIVED PEPTIDES

Dodecamer (Amino acids 18-29 of saposin C; 12 amino acid internal peptide fragment)
SEQ ID NO:4
LeuIleAspAsnAsnLysThrGluLysGluIleLeu

Octadecamer (18 amino acid peptide fragment)
SEQ ID NO:5
TyrLysGluValThrLysLeuIleAspAsnAsnLysThrGluLysGluIleLeu

Docosanamer (22 amino acid peptide fragment)
SEQ ID NO:6
CysGluPheLeuValLysGluValThrLysLeuIleAspAsnAsnLysThrGluLysGluIleLeu

Tetradecamer (Prosaptide TX14(A))
SEQ ID NO:7
ThrDAlaLeuIleAspAsnAsnAlaThrGluGluIleLeuTyr

Mouse Peptide
SEQ ID NO:8
CysGlnPheValMetAsnLysPheSerGluLeuIleValAsnAsnAlaThrGluGluLeuLeuTyr

Rat Prosaptide
SEQ ID NO:9
CysGlnLeuValAsnArgLysLeuSerGluLeuIleIleAsnAsnAlaThrGluGluLeuLeu

Guinea Pig Prosaptide
SEQ ID NO:10
CysGluTyrValValLysLysValMetLeuLeuIleAspAsnAsnArgThrGluGluLysIleIle

Bovine Prosaptide
SEQ ID NO:11
CysGluPheValValLysGluValAlaLysLeuIleAspAsnAsnArgThrGluGluGlu IleLeu

Rat Prosaptide
SEQ ID NO:12
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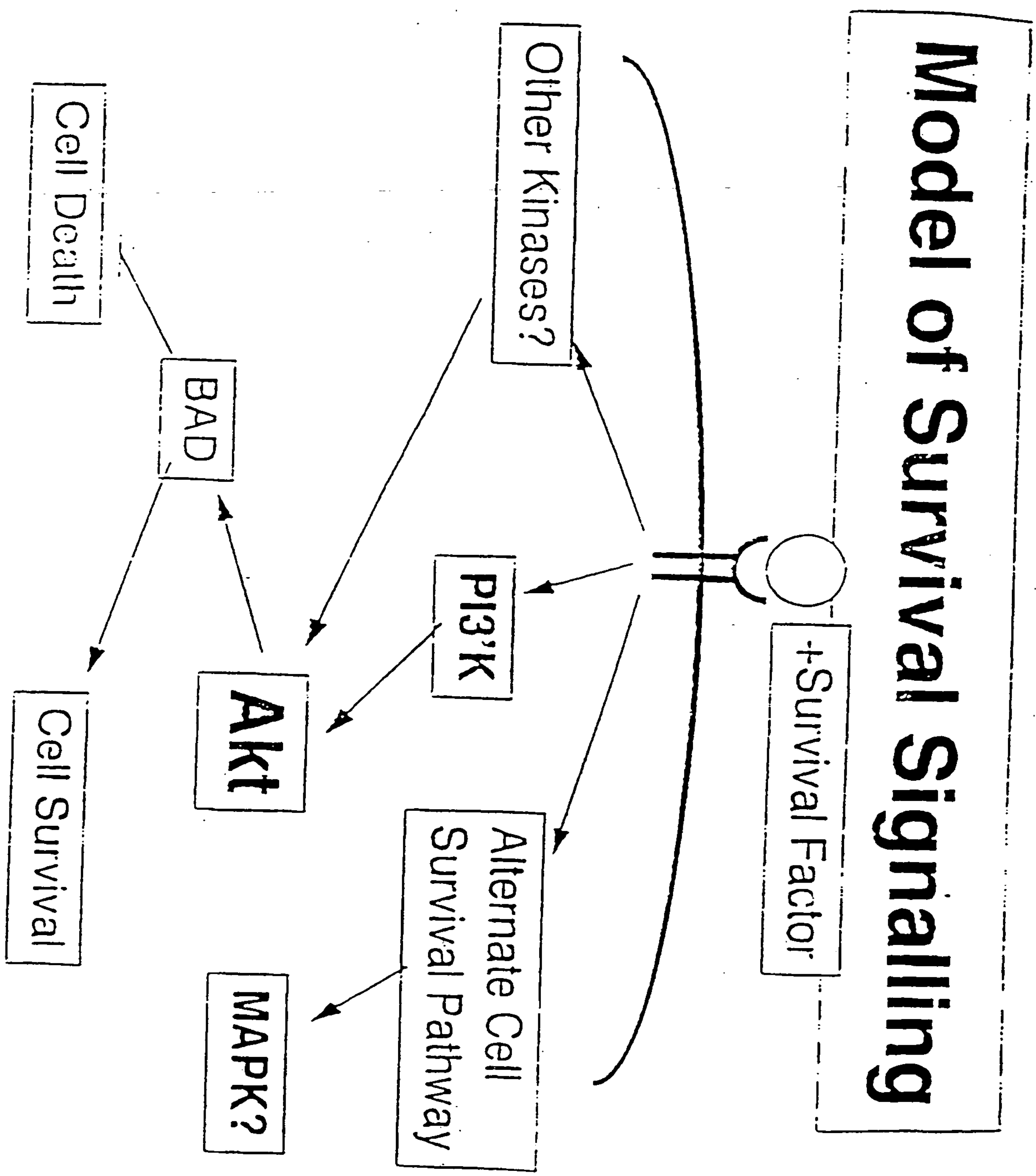
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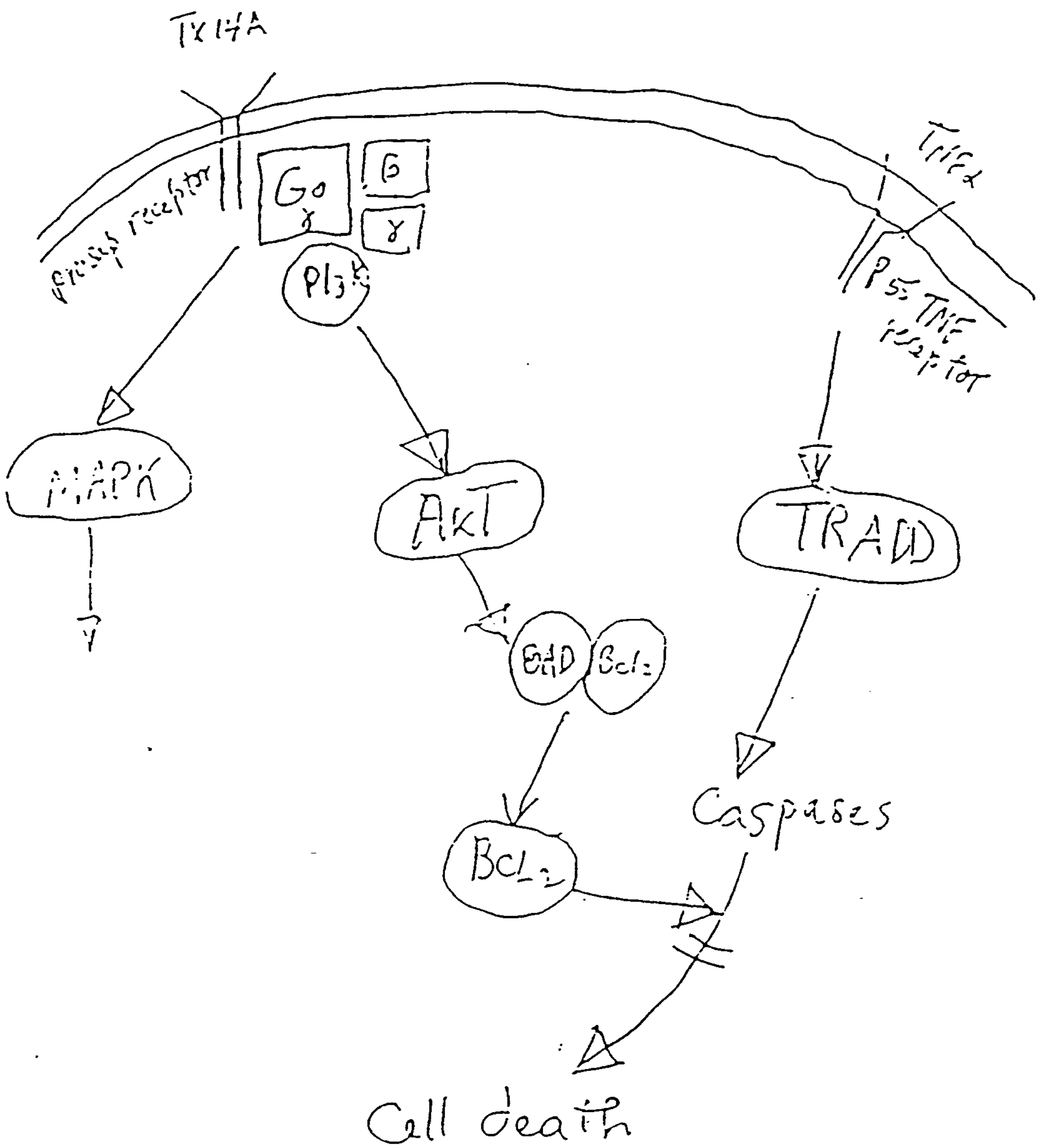
Other Kinases?
PI3'K
Alternate Cell Survival Pathway
Akt
MAPK?
BAD
Cell Death
Cell Survival

FIGURE 4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prosap receptor
Go
γ
β
γ
PI3K
TNFα
P55 TNF receptor
MAPK
AkT
TRADD
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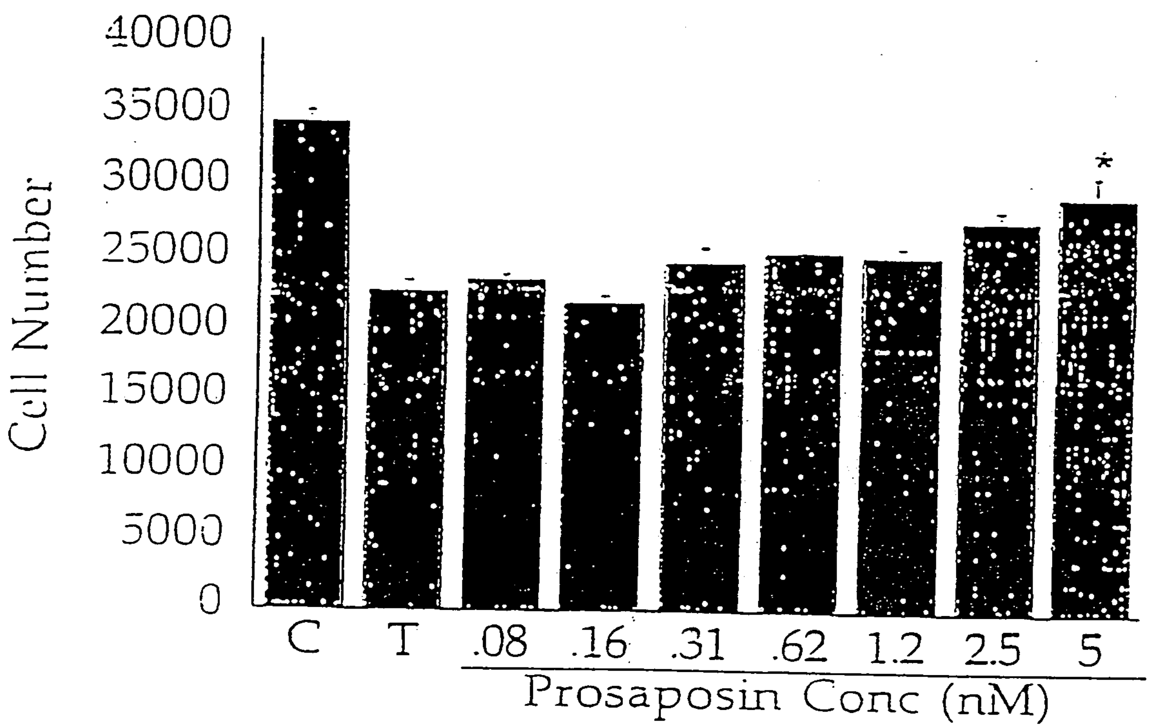
Bcl2
BcL2
Caspases
Cell death

FIGURE 5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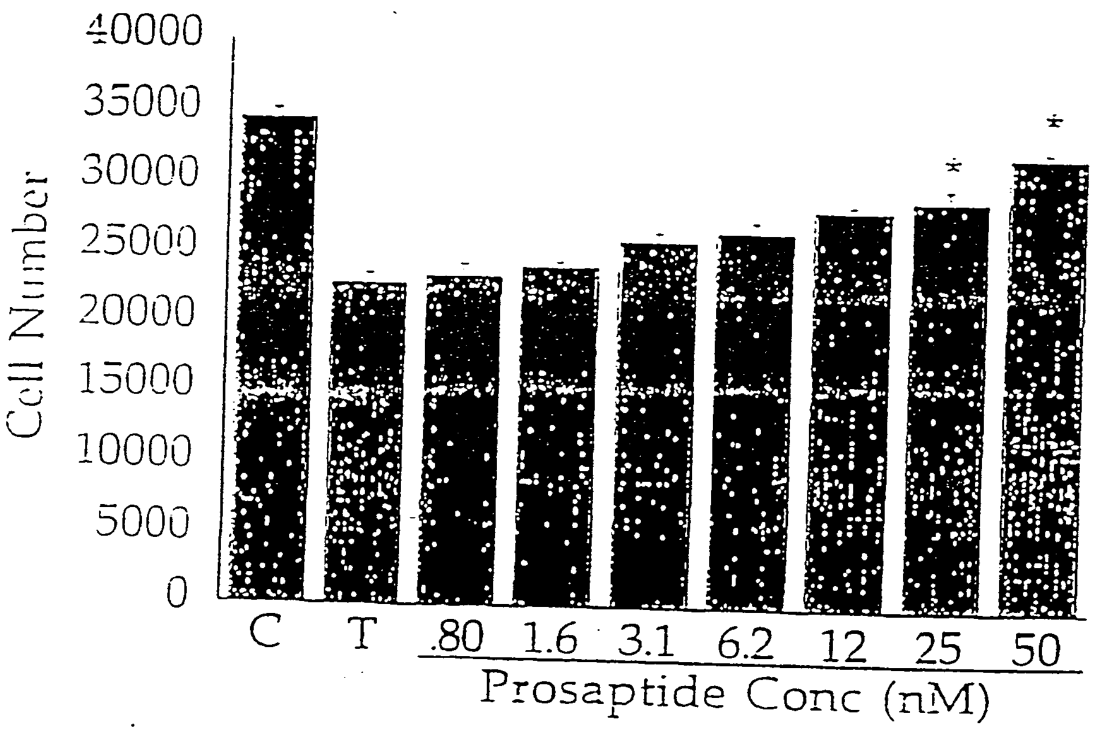
Prosaposin Conc (nM)

FIGURE 6A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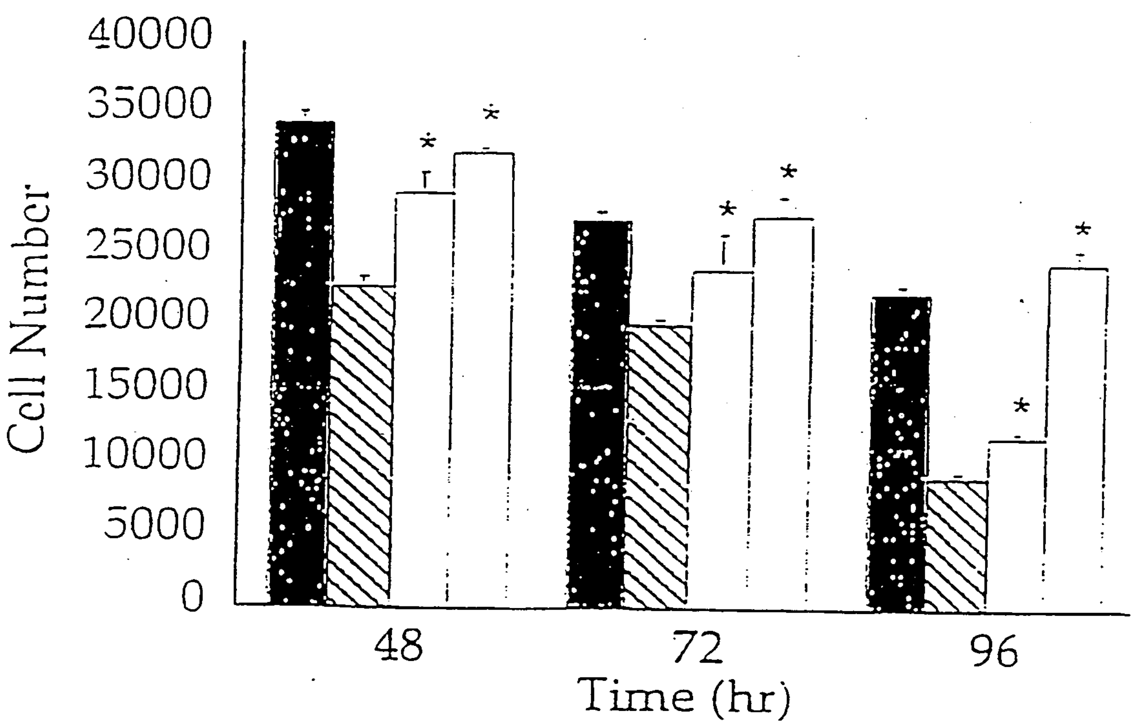
Prosaptide Conc (nM)

Figure 6B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*
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72
96
Time (hr)

Figure 7

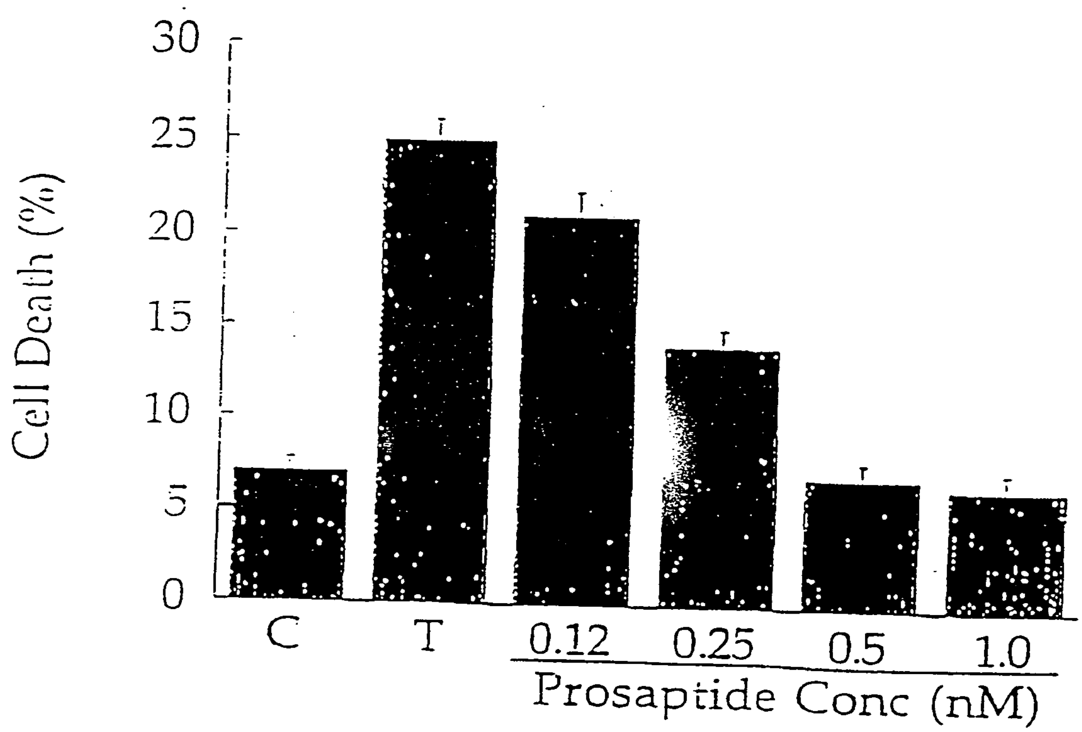


Figure 8

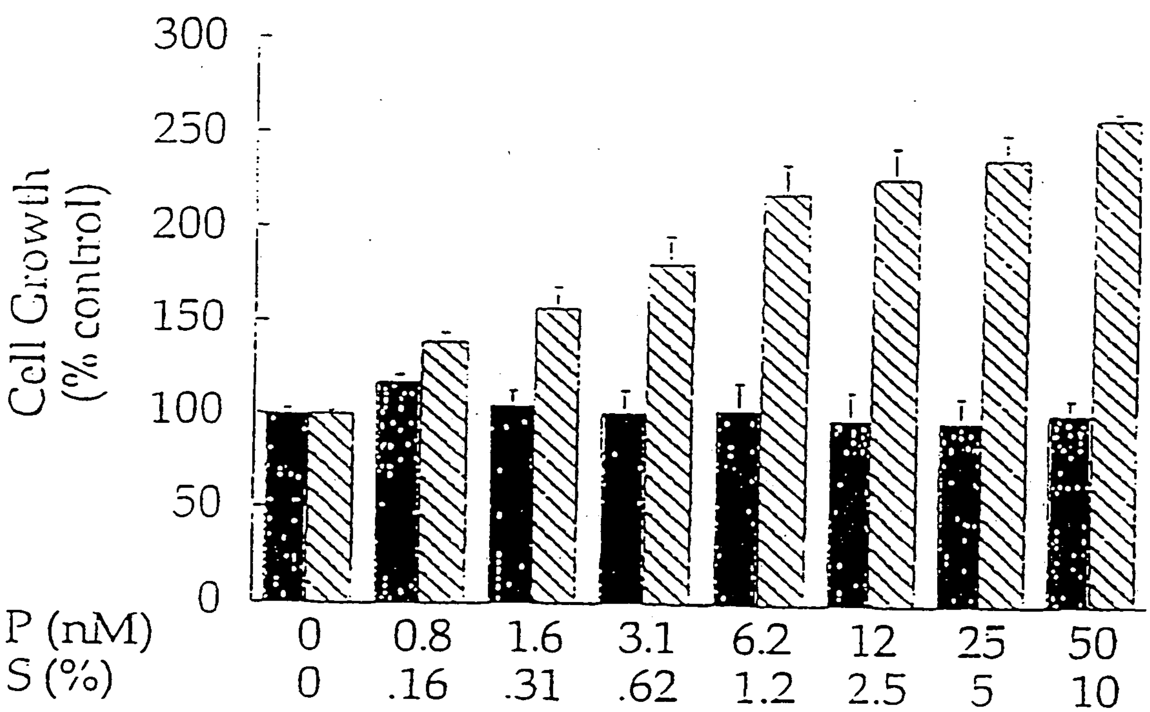

Cell Growth
(% control)
300
250
200
150
100
50
0
P (nM)
S (%)
0
0
0.8
.16
1.6
.31
3.1
.62
6.2
1.2
12
2.5
25
5
50
10

Figure 9

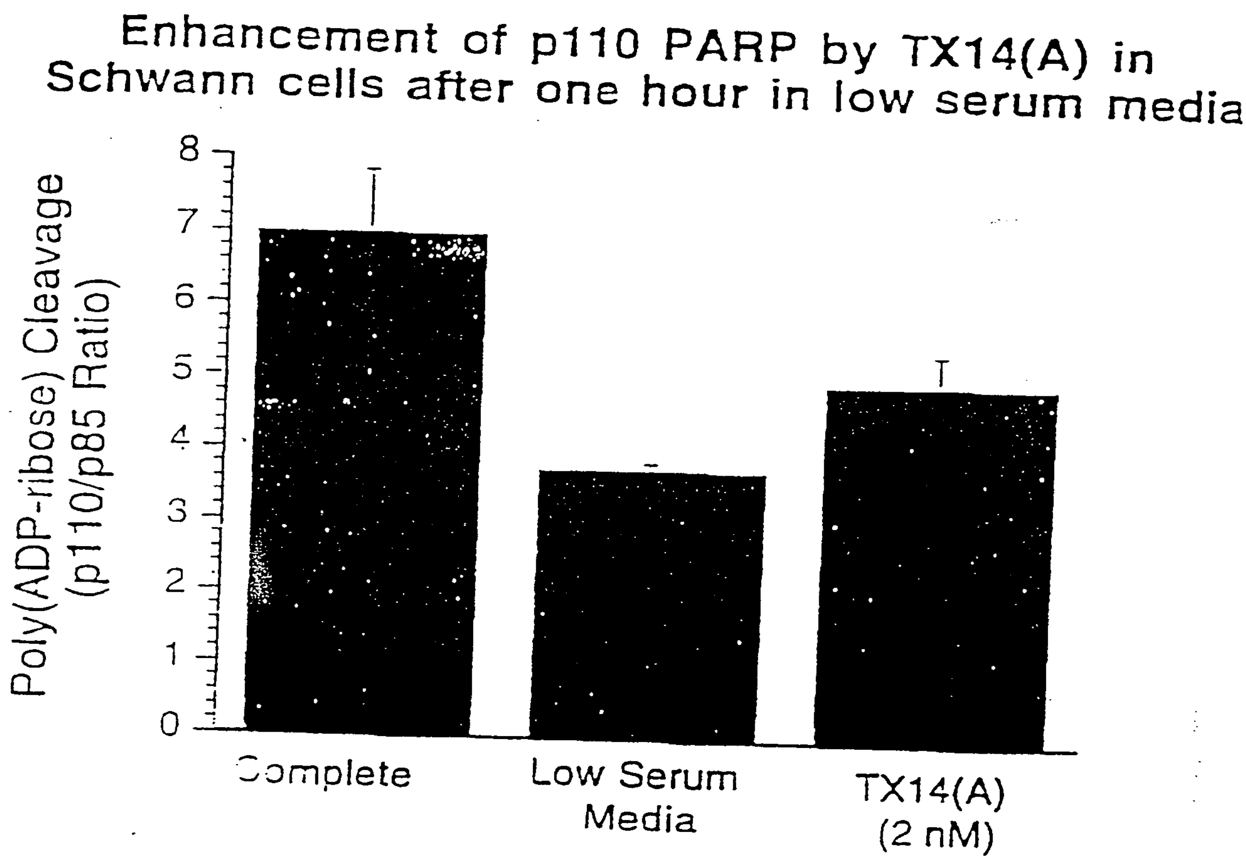

Enhancement of p110 PARP by TX14(A) in
Schwann cells after one hour in low serum media
Poly(ADP-ribose) Cleavage
(p110/p85 Ratio)
8
7
6
5
4
3
2
1
0
Complete
Low Serum
Media
TX14(A)
(2 nM)

Figure 10

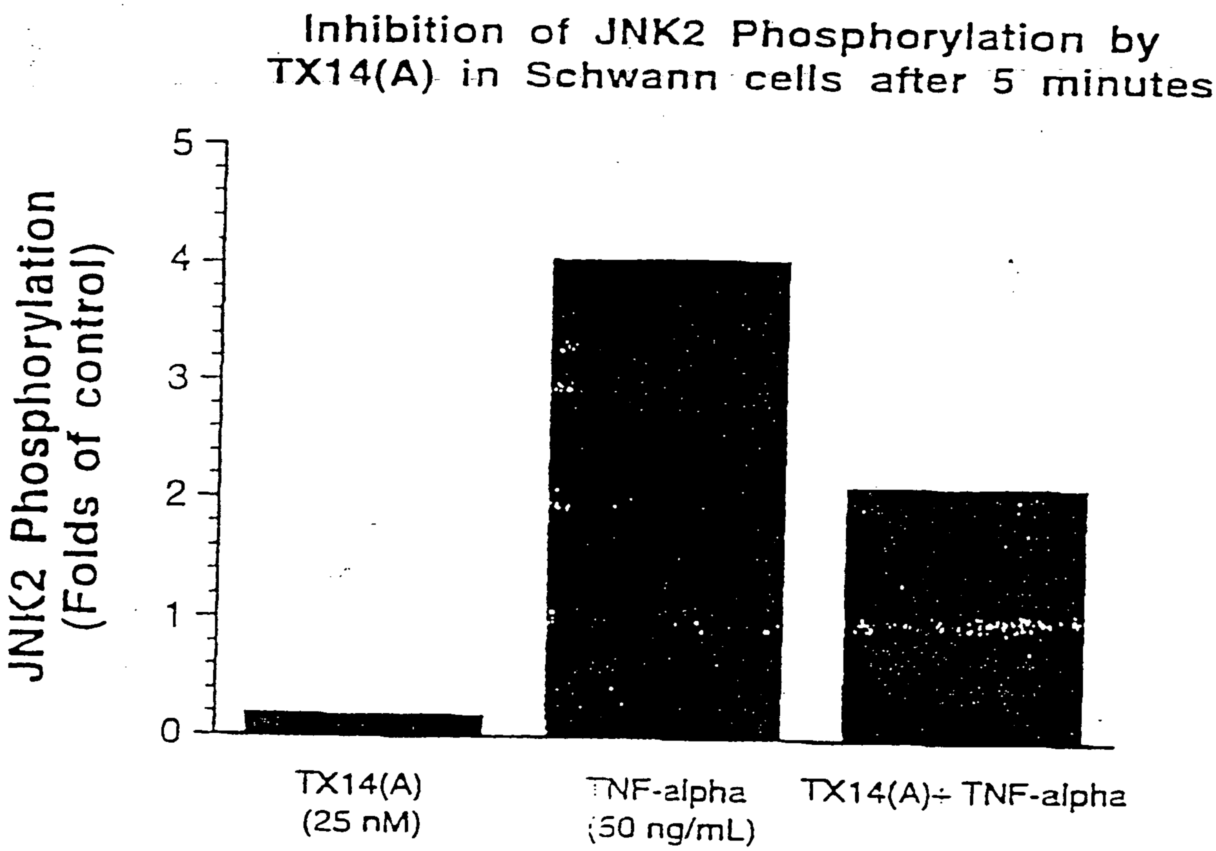

Inhibition of JNK2 Phosphorylation by TX14(A) in Schwann cells after 5 minutes
JNK2 Phosphorylation (Folds of control)
0
1
2
3
4
5
TX14(A) (25 nM)
TNF-alpha (50 ng/mL)
TX14(A)+ TNF-alpha

Figure 11

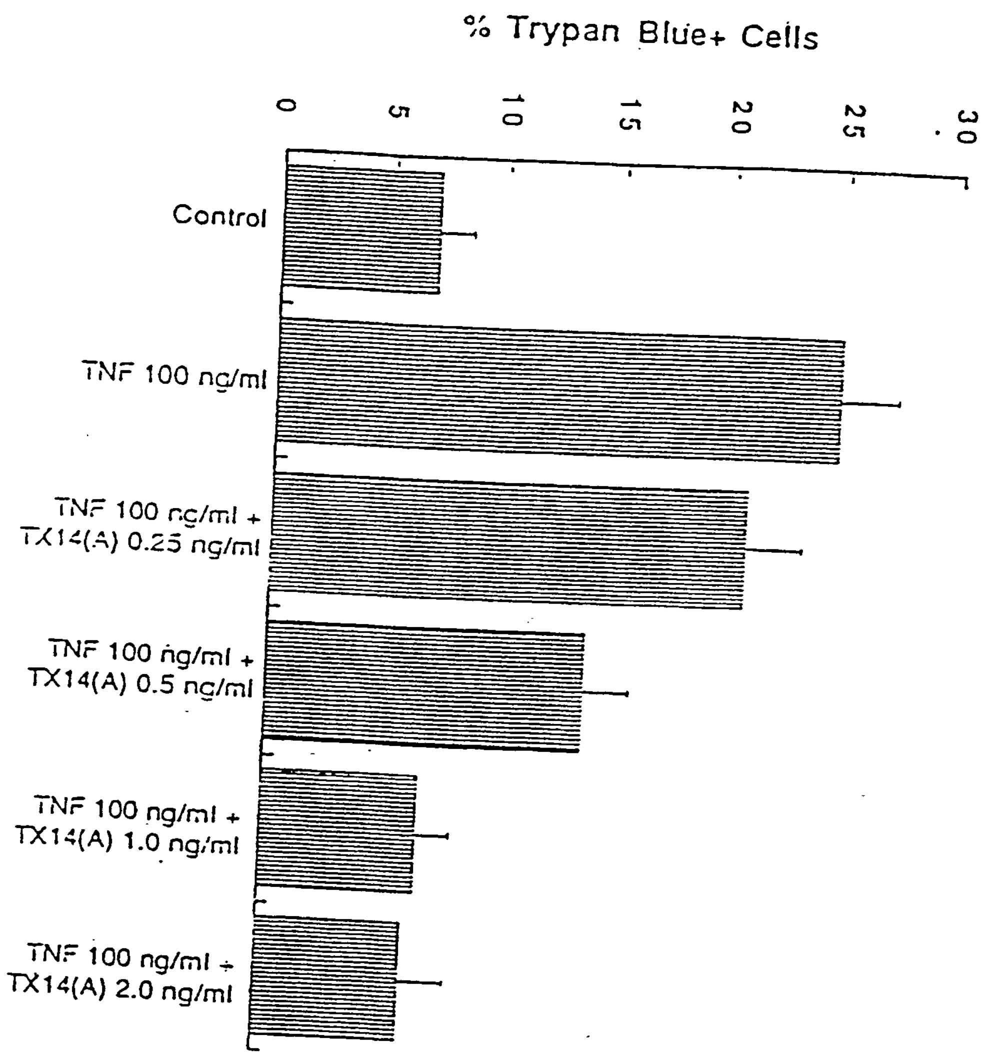
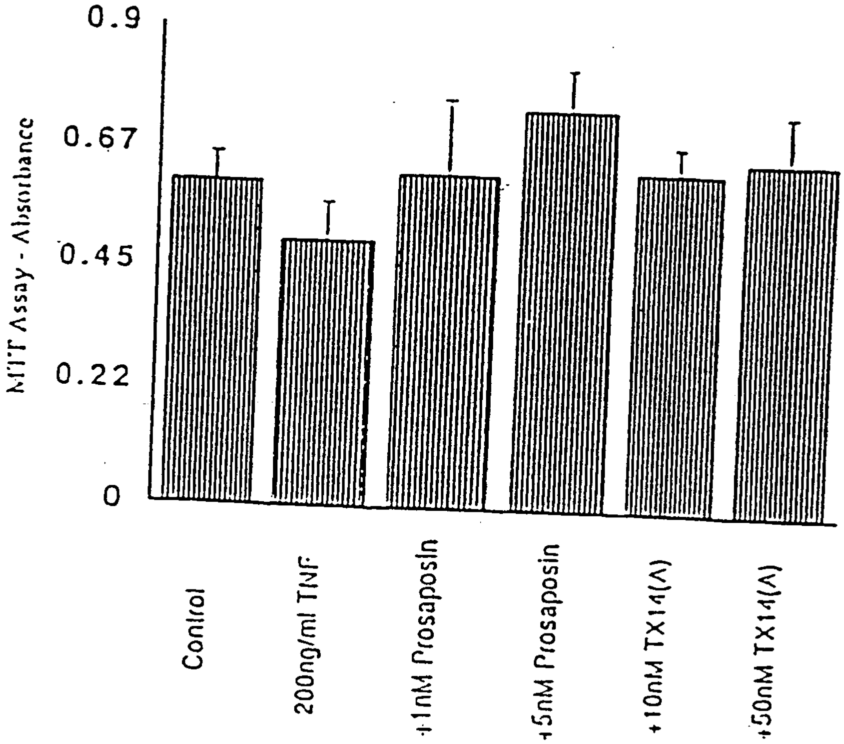

% Trypan Blue+ Cells
0
5
10
15
20
25
30
Control
TNF 100 ng/ml
TNF 100 ng/ml +
TX14(A) 0.25 ng/ml
TNF 100 ng/ml +
TX14(A) 0.5 ng/ml
TNF 100 ng/ml +
TX14(A) 1.0 ng/ml
TNF 100 ng/ml +
TX14(A) 2.0 ng/ml

FIGURE 12


MTT Assay - Absorbance
0.9
0.67
0.45
0.22
0
Control
200ng/ml TNF
+1nM Prosaposin
+5nM Prosaposin
+10nM TX14(A)
+50nM TX14(A)

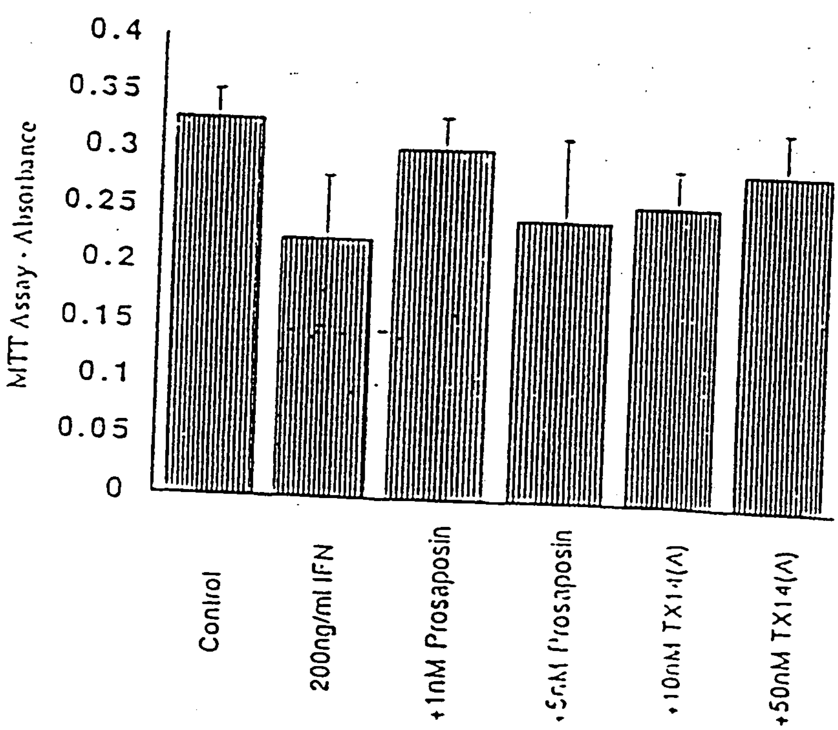

MTT Assay - Absorbance
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
200ng/ml IFN
+1nM Prosaposin
+5nM Prosaposin
+10nM TX14(A)
+50nM TX14(A)

FIGURE 13

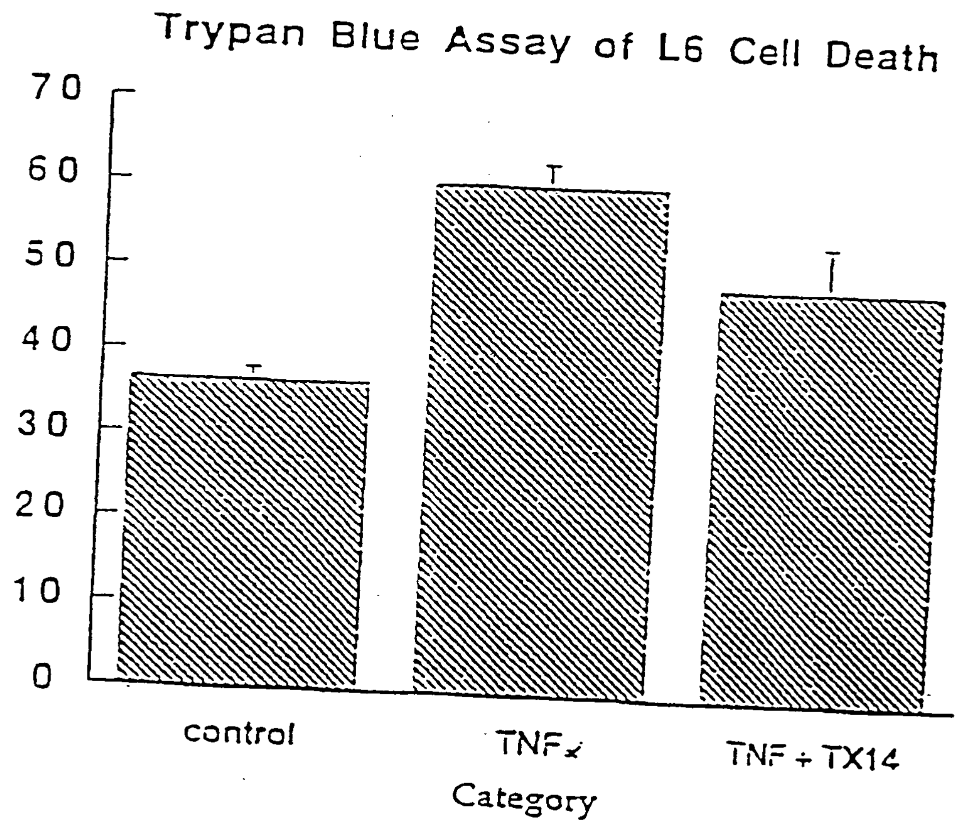

Trypan Blue Assay of L6 Cell Death
70
60
50
40
30
20
10
0
control
TNFα
TNF + TX14
Category

FIGURE 14

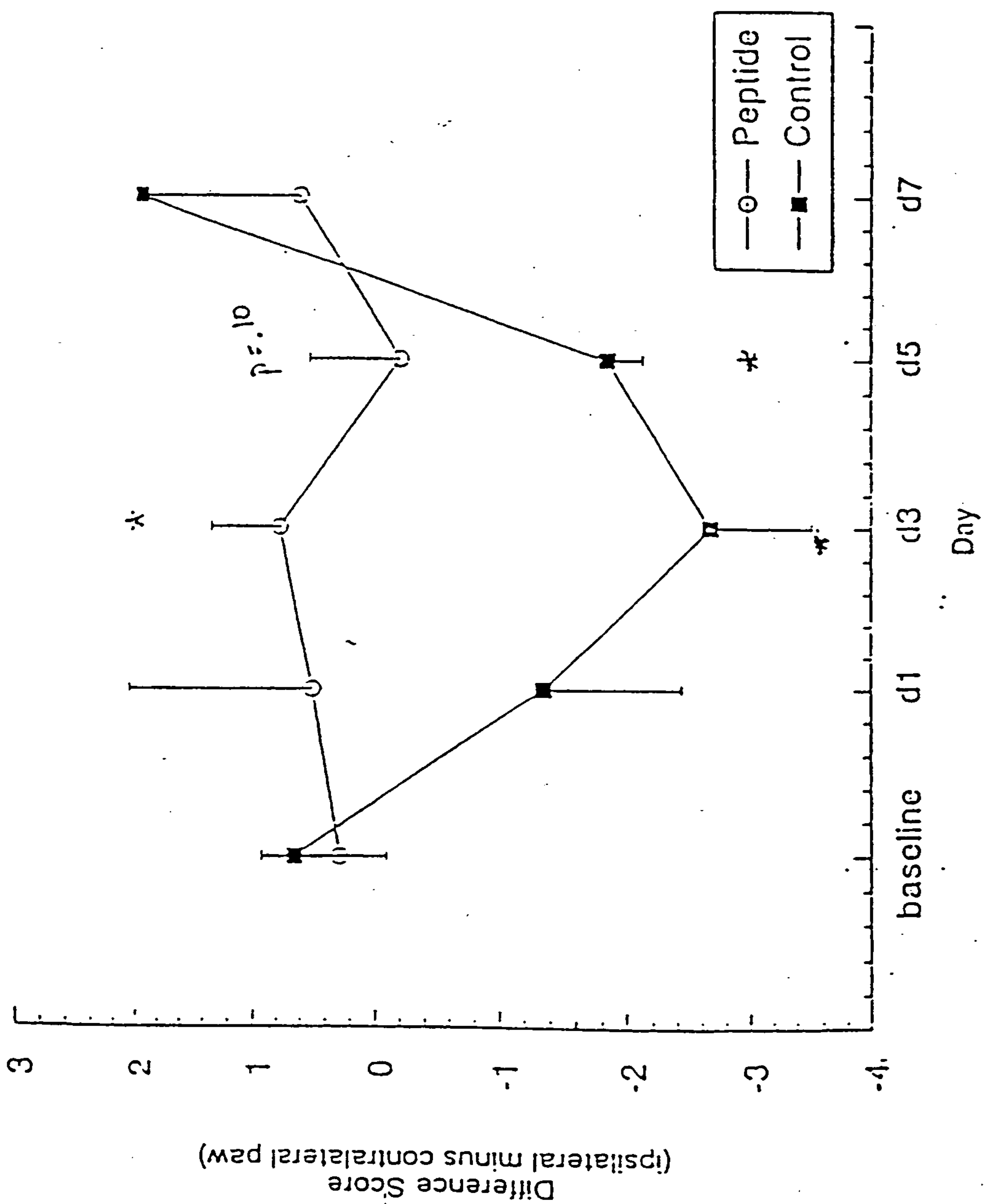

Peptide
Control
p=.10
*
baseline
d1
d3
d5
d7
Day
3
2
1
0
-1
-2
-3
-4
Difference Score
(ipsilateral minus contralateral paw)

FIGURE 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19216

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/04, 38/10, 38/16, 38/19
US CL : 514/12, 13, 14; 530/324, 325, 326, 327
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 13, 14; 530/324, 325, 326, 327

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P ------ X,P	WO 97/32895 A1 (REGENTS OF THE UNIVERSITY OF CALIFORNIA) 12 September 1997, entire document, especially Abstract.	1-11 -------- 11-16
A	WO 95/03821 A1 (REGENTS OF THE UNIVERSITY OF CALIFORNIA) 09 February 1995, entire document.	1-16

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 DECEMBER 1998	29 JAN 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GEETHA P. BANSAL D. Lawrence for Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19216

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

CAPLUS, BIOSIS, MEDLINE, EMBASE, USPATFULL, WPIDS, PROTEIN SEQUENCE DATABASE
search terms: prosaposin, prosaposin receptor, apoptosis, caspase